The Journal of Experimental Biology 202, 485–495 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JEB1850

PLASMODIUM GALLINACEUM OOKINETES ADHERE SPECIFICALLY TO THE MIDGUT EPITHELIUM OF *AEDES AEGYPTI* BY INTERACTION WITH A CARBOHYDRATE LIGAND

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Accepted 9 December 1998; published on WWW 3 February 1999

Summary

During the course of its development in the mosquito and transmission to a new vertebrate host, the malaria parasite must interact with the mosquito midgut and invade the gut epithelium. To investigate how the parasite recognizes the midgut before invasion, we have developed an *in vitro* adhesion assay based on combining fluorescently labelled ookinetes with isolated midgut epithelia from blood-fed mosquitoes. Using this assay, we found that *Plasmodium gallinaceum* ookinetes readily adhered to midguts of *Aedes aegypti*, mimicking the natural recognition of the epithelium by the parasite. This interaction is specific: the ookinetes preferentially adhered to the lumen (microvillar) side of the gut epithelium and did not bind to other mosquito tissues. Conversely, the binding was not due to a non-specific adhesive property of the midguts, because a variety of other cell types, including untransformed *P. gallinaceum* zygotes or macrogametes, did not show similar binding to the midguts. High concentrations of glycosylated (fetuin, orosomucoid, ovalbumin) or non-glycosylated (bovine serum albumin) proteins, added as non-specific competitors, failed to compete with the ookinetes in binding assays. We also found that the adhesion of ookinetes to the midgut surface is necessary for sporogonic development of the parasite in the mosquito. Antibodies and other reagents that blocked adhesion *in vitro* also reduced oocyst formation when these reagents were combined with mature ookinetes and fed to mosquitoes. Chemical modification of the midguts with sodium periodate at pH 5.5 destroyed adhesion, indicating that the ookinete binds to a carbohydrate ligand on the surface of the midgut. The ligand is sensitive to periodate concentrations of less than 1 mmol l^{-1}, suggesting that it may contain sialic-acid-like sugars. Furthermore, free *N*-acetylneuraminic acid competed with the ookinetes in binding aasays, while other monosaccharides had no effect. However, in agreement with the current belief that adult insects do not contain sialic acids, we were unable to detect any sialic acids in mosquito midguts using the most sensitive HPLC-based fluorometric assay currently available. We postulate that a specific carbohydrate group is used by the ookinete to recognize the midgut epithelium and to attach to its surface. This is the first receptor–ligand interaction demonstrated for the ookinete stage of a malaria parasite. Further characterization of the midgut ligand and its parasite counterpart may lead to novel strategies of blocking oocyst development in the mosquito.

Key words: malaria, mosquito, *Aedes aegypti*, midgut, transmission blocking, insect, carbohydrate, sialic acid, *Plasmodium gallinaceum*.

Introduction

Malaria is a widespread, mosquito-transmitted tropical disease. Despite a century of effort, effective and cheap methods of malaria control do not yet exist. The most promising new approaches seek to disrupt the transmission cycle in natural populations of vector mosquitoes *via* transmission-blocking vaccines or controlled release of parasite-refractory strains of mosquito (Crampton et al., 1994; Gwadz, 1994; Collins and Paskewitz, 1995). However, designing an effective transmission blocking strategy requires a full molecular understanding of the sporogonic development of the parasite in the mosquito (Billingsley, 1994).

The vector mosquito acquires the *Plasmodium gallinaceum* parasite through an infected blood meal. The malaria parasite undergoes a complex development in the midgut of the mosquito and becomes a motile ookinete, which matures in the blood meal within 24 h of ingestion. The mature ookinete then leaves the blood meal by crossing the peritrophic membrane, makes contact with the microvillar surface of the midgut

epithelium and penetrates the midgut cells. After crossing the epithelium, it reaches the basement membrane separating the midgut from the haemolymph of the insect, where it changes into an oocyst. Sporozoites develop within the oocyst, are released and migrate to the salivary glands of the mosquito. They are injected into the bloodstream of a new vertebrate host during feeding to begin a new cycle of infection.

Ookinetes do not have the capacity to bind to and invade a wide range of cell types (Rosales-Ronquillo et al., 1974; Rosales-Ronquillo and Silverman, 1974), but specifically interact with mosquito midgut tissue (Shahabuddin and Pimenta, 1998). Since initial attachment of microbes or parasites to their host tissue is often necessary for invasion (Karlsson, 1989, 1995; Finlay, 1990; Rostand and Esko, 1997), we postulated that the ookinete may recognize the midgut surface by specific molecular interactions before invasion of the epithelium. This first contact and recognition may be crucial for the efficient penetration of the midgut epithelium.

Adhesion to specific cell types and invasion of specific tissues are recurring themes in the life cycle of *Plasmodium gallinaceum*. The interactions between the mammalian stages of the parasite and their host have been studied extensively, and a great deal is known about receptor proteins on the parasite surface and ligands on the target tissue. Sporozoites injected into the bloodstream by a feeding mosquito bind to heparan sulphate proteoglycans on the surface of hepatocytes in the space of Disse of the liver and proceed to invade the hepatocytes (Frevert et al., 1993; Frevert, 1994). Merozoites released from a red blood cell bind to glycoproteins on other red blood cells *via* terminal sialic acid on N-linked carbohydrates (Hadley and Miller, 1988; Holt et al., 1989; Adams et al., 1992; Klotz et al., 1992; Orlandi et al., 1992; Pasvol et al., 1993; Satapathy et al., 1993; Dolan et al., 1994; Sim et al., 1994); this initial attachment of merozoites to red blood cells is thought to be mediated by MSP-1, the major surface molecule of the merozoite (Perkins and Rocco, 1988; DeLuca et al., 1996). Invasion of the red blood cells by *P. falciparum* can occur by a sialic-acid-dependent pathway that is mediated by EBA175, a merozoite protein necessary for the invasion of a red blood cell (Camus and Hadley, 1985; Klotz et al., 1992; Orlandi et al., 1992; Sim et al., 1994). Parasite-infected red blood cells adhere to the vascular endothelium of various organs by making specific contact with a variety of adhesion proteins or chondroitin sulphate glycans (Berendt et al., 1994; Rogerson et al., 1995, 1997; Cooke et al., 1996; Fried and Duffy, 1996; Gysin et al., 1997).

In contrast, few attempts have been made to characterize the interactions between the insect stages of the malaria parasite and mosquito tissues. The description of ookinete penetration of the midgut epithelium has been limited to studies by light and electron microscopy (Mehlhorn et al., 1980; Meis and Ponnudurai, 1987; Meis et al., 1989; Sieber et al., 1991; Syafruddin et al., 1991; Torii et al., 1992). These studies primarily addressed the events occurring between ookinete invasion of the midgut epithelium and oocyst formation, and the molecular details of these processes are not known.

We recognized from reviewing the literature on merozoite invasion of red blood cells and sporozoite invasion of cultured hepatocytes that *in vitro* assays are indispensable for the molecular characterization of adhesion and invasion processes. It is possible to prepare *P. gallinaceum* ookinetes *in vitro* (Carter et al., 1979; Kaushal et al., 1983). We recently showed that, when pure ookinetes are combined with isolated midgut epithelia from blood-fed mosquitoes, they bind to the epithelium in large numbers. Subsequently, some of the parasites invade midgut cells and penetrate through the epithelium as they do in naturally infected mosquitoes (Shahabuddin and Pimenta, 1998). In the present study, we have modified this simple procedure to provide a quantitative *in vitro* assay of ookinete adhesion to mosquito midguts.

We report the results of the first attempts to characterize the adhesive interaction between ookinetes and the mosquito midgut. We show that ookinetes adhere specifically to the luminal surface of the midgut and that this adhesion is required for normal levels of oocyst formation *in vivo*. We also provide evidence that the ligand on the midgut surface is a carbohydrate that shares biochemical characteristics with sialic acids.

Materials and methods

Parasites and mosquitoes

The Liverpool/black eye strain of *Aedes aegypti* and the 8A strain of *Plasmodium gallinaceum* were used throughout this study. Mosquitoes were raised and fed using standard techniques (Gerberg et al., 1994; Higgs and Beaty, 1996). Parasites were maintained in white Leghorn chickens by serial blood passages.

Purification and staining of Plasmodium gallinaceum *ookinetes*

Plasmodium gallinaceum zygotes were purified from the processed blood of infected white Leghorn chickens (Carter et al., 1979; Kaushal et al., 1983). Chickens with a parasitaemia of 25–75 % were bled by cardiac puncture, the blood was heparinized in the syringe and immediately added to a fourfold excess of 1× suspended animation medium (1× SA: 9 mmol l^{-1} glucose, 8 mmol l^{-1} Tris base, 138 mmol l^{-1} sodium chloride, pH 7.3) prewarmed to 39 °C. The blood cells were pelleted by centrifugation at 2500 revs min^{-1} in a table-top centrifuge at room temperature, and were then resuspended in the original blood volume of exflagellation medium (75 ml of 1× SA, 15 ml of 1.5 % sodium bicarbonate, 8 ml of filtered, heat-inactivated chicken serum and 2 ml of 5 mmol l^{-1} xanthurenic acid, pH 8.0). Exflagellation and mating of male and female gametes was allowed to proceed by letting the mixture stand for 30 min at room temperature (20–25 °C). The mixture was then layered on top of a 15 ml cushion of a Ficoll/Hypaque mixture in a sterile 50 ml plastic centrifuge tube and centrifuged at 3500 revs min^{-1} for 15 min in a swinging-bucket rotor. The Ficoll/Hypaque solution contained 24 ml of 9 % Ficoll 400 (from Sigma, St Louis, MO, USA, dissolved in distilled water) and 15 ml of 34 % Hypaque (Nycomed Inc., New York, USA,

diluted in distilled water). The cell layer at the interface was removed, pelleted and resuspended in 10 ml of 1× SA. To agglutinate the remaining chicken cells, 0.7 ml of wheat germ agglutinin (1 mg ml^{-1}, Sigma) was added and mixed thoroughly with the cells, which were then allowed to stand for 10 min. Low-speed centrifugation of the tube at 500 revs min^{-1} for 1 min pelleted any remaining agglutinated chicken cell clumps but left the parasites (*Plasmodium gallinaceum* macrogametes and zygotes) in suspension. The supernatant was transferred to a fresh tube and the parasites were pelleted by centrifugation at 2500 revs min^{-1} for 5 min. The purified parasites were resuspended in M199 medium supplemented with 2 mmol l^{-1} L-glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (ookinete medium) at a density of 2×10^6 to 5×10^6 zygotes ml^{-1} and incubated for 16–20 h at 26 °C to allow development of ookinetes. For fluorescent staining of the parasites with the cell marker PKH26 (Shahabuddin et al., 1997), the fully developed ookinetes were concentrated by centrifugation, washed once with 0.5 ml of 5.4 % glucose and stained for 1 min in 0.4 ml of 125 μmol l^{-1} PKH26 red fluorescent cell linker (Sigma, St Louis, MO, USA) in 5.4 % glucose. The ookinetes were then washed three times with 0.8 ml of ookinete medium supplemented with 10 % heat-inactivated chicken serum (adhesion medium).

Isolation of mosquito midgut epithelia

We used midgut epithelia from blood-fed mosquitoes in our experiments because they correspond exactly to the tissue invaded by ookinetes *in vivo*. To prepare midgut epithelia, female *Aedes aegypti* mosquitoes were fed on uninfected chickens. Twenty-four hours after feeding, the mosquitoes were collected by aspiration, anaesthetized by cooling on ice and their midguts were extracted. The anterior midgut, hindgut and Malpighian tubules were removed, and the posterior midgut containing the blood meal was cut in half lengthwise using a scalpel. The midgut wall, which spontaneously peels away from the blood meal, was gently separated from the peritropic matrix using dissection needles to leave an isolated epithelium. The resulting half-gut epithelia are referred to as gut sheets. Each pair of gut sheets was collected in a separate Eppendorf tube. All dissections were performed in adhesion medium (M199 medium supplemented with 2 mmol l^{-1} L-glutamine, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10 % heat-inactivated chicken serum). When needed, midguts were treated with a variety of modifying reagents as described below. After treatment, the midgut sheets were thoroughly washed and re-equilibrated with adhesion medium.

Assay of ookinete binding to the midgut epithelium

Pairs of midgut sheets were suspended in 100 μl of adhesion medium in 1.5 ml Eppendorf tubes. Ookinete suspension (50 μl) was added (a total of 10^5 to 3×10^5 ookinetes). Other reagents were added if necessary, and a uniform pH was maintained for all adhesion reactions. Ookinetes and midgut sheets were brought together by centrifuging the mixture three times for 1 min at 300 ***g*** in an Eppendorf microfuge. This allows the ookinetes to come into contact with the midgut and to adhere. Following each centrifugation, the gut sheets and ookinetes were resuspended by flicking the tubes gently. After the last resuspension, the gut sheets were washed twice with 1 ml of adhesion medium and placed on ice. Each pair of sheets was transferred to a glass slide, and the midguts were gently spread out into flat sheets using dissection needles. They were then fixed with 0.1 mol l^{-1} sodium cacodylate, 0.1 mol l^{-1} sucrose, 2.5 % glutaraldehyde and 4 % paraformaldehyde at pH 7.0, covered with a coverslip and examined under a fluorescence microscope to detect and quantify bound ookinetes. Typically, between 200 and 1000 ookinetes adhere to a single gut sheet. Each adhesion experiment reported in this paper was performed with quadruplicate samples. The results of typical experiments are presented in the figures, but each experiment was repeated at least twice, and comparable results were obtained each time. Statistical significances were calculated using the *t*-test.

Periodate and glycosidase treatments of mosquito midgut epithelia

Periodate treatments involved washing midgut sheets once with 0.5 ml of 50 mmol l^{-1} sodium acetate, 100 mmol l^{-1} NaCl, pH 5.5, and then incubating them in various concentrations of sodium meta-periodate in the same buffer for 30 min on ice in the dark. *N*-glycosidase A and *O*-glycosidase were obtained from Boehringer Mannheim (Indianapolis, IN, USA) and Endoglycosidase-F/Peptide-*N*-glycosidase F was obtained from Oxford Glycosystems (Abington, UK). Midgut sheets were treated with *N*-glycosidase A in 50 mmol l^{-1} sodium acetate, 100 mmol l^{-1} NaCl at pH 6.0 (50 μl total volume) for 1 h at room temperature. Endo-F/Peptide-*N*-glycosidase F and *O*-glycosidase treatments were performed in phosphate-buffered saline (PBS). The following amounts of enzyme were used for treatment of one pair of gut sheets in a total volume of 50 μl: *N*-glycosidase A, 1 milliunit; Endo-F/Peptide-*N*-glycosidase F, 5 units; *O*-glycosidase, 5 milliunits. All enzyme units are defined as specified by the manufacturers. Protease inhibitors were included in all enzymatic midgut treatments. The glycoproteins used in adhesion assays, bovine serum albumin (BSA), bovine fetuin, bovine orosomucoid and chicken ovalbumin, and all the monosaccharides were bought from Sigma (St Louis, MO, USA).

Preparation of total mosquito carbohydrate

Total mosquito carbohydrate was prepared from 20 g of adult *Aedes aegypti* using a method similar to that already published (Finne and Krusius, 1982). The mosquitoes were frozen in liquid N_2 and ground to a fine powder in a porcelain mortar on dry ice. The powder was then delipidated by serial extractions with chloroform/methanol 1:1, and the residue was dried, triturated with acetone, dissolved in distilled water and subjected to prolonged pronase digestion. The resulting solution was then dialyzed (10 kDa molecular mass cut-off), and the high-molecular-mass material was lyophilized, weighed and redissolved in distilled water.

Transmission blocking assays

P. gallinaceum transmission blocking assays were performed as described previously (Sieber et al., 1991). Total mosquito carbohydrate (approximately 70 mg ml^{-1}) or the H.46 horse polyclonal antiserum directed against the K1 strain of *Escherichia coli* (Troy et al., 1987) was diluted 1:10 in chicken blood that had been washed twice with heat-inactivated chicken serum. Ookinetes were mixed into the serum-containing chicken blood at a final concentration of 10^7 ookinetes ml^{-1} and were fed to female *Aedes aegypti* mosquitoes as described previously (Sieber et al., 1991; Higgs and Beaty, 1996). Eight days after feeding, midguts were removed from gravid females, stained with mercurichrome and examined for oocyst numbers. The same 1:10 dilution of carbohydrate and antisera was used for adhesion-blocking experiments. To test for their effects on ookinete viability, either reagent was mixed into an ookinete suspension and incubated for 2 h. The ookinetes were then stained with ethidium homodimer, as described elsewhere, to determine the percentage of non-viable parasites (Shahabuddin et al., 1998).

Preparation of midgut acid hydrolysates

A crude acid hydrolysate of mosquito midguts was prepared as follows. Midguts were removed from fed or unfed mosquitoes in sterile PBS and collected in a small volume of PBS on ice. For several days prior to the removal of their midguts, all mosquitoes had been fed a solution of 0.2 mol l^{-1} glucose with antibiotics to eliminate midgut microbial populations. The midguts were washed several times with PBS and pelleted. The midgut pellet was homogenized in distilled water and acid-hydrolyzed under various conditions: 0.1 mol l^{-1} trifluoroacetic acid (TFA) for 0.5–2 h at 80 °C (Stanton et al., 1995); 0.5 mol l^{-1} formic acid for 1–2 h at 80 °C; 2 mol l^{-1} acetic acid for 1–3 h at 80 °C. After hydrolysis, the samples were centrifuged to pellet the cell debris, and the supernatant was centrifuged through a Microcon 3 ultrafiltration device (Amicon Inc., Beverly, MA, USA) to remove molecules larger than 3000 Da. The low-molecular-mass flow-through was passed through a Sep-Pak Plus C18 column (Waters Corp., Milford MA, USA), which was washed with one column volume of distilled water. The column flow-through and water wash were pooled, dried in a Speed-Vac and redissolved in distilled water.

Sialic acid assays

1,2-Diamino-4,5-methylenedioxybenzene (DMB) was bought from Dojindo Laboratories (Tokyo, Japan). Fluorometric DMB assays for derivatizing midgut hydrolysates and detecting them by fluorescence were carried out as described elsewhere (Hara et al., 1987; Klein et al., 1997). Following the reaction of the midgut extracts with DMB, reaction samples were chromatographed by HPLC on an TosoHaas (Montgomeryville, PA, USA) ODS-120T column (25 cm×4.1 mm) and eluted with a 40 min gradient of 7 % methanol, 7 % acetonitrile to 7 % methanol, 11 % acetonitrile. Fluorescent peaks were detected with a Thermo Separation Products (San José, CA, USA) FL3000 fluorescent detector. Using this method, we were able to detect less than 1 pmol of *N*-acetylneuraminic acid (NANA) or the related sugars keto-deoxynononic acid (KDN) and keto-deoxyoctonic acid (KDO); NANA could also be detected, with minimal loss of material, when added to mosquito midguts before acid hydrolysis and carried through the entire purification procedure described above. For mass spectrometric analysis (Klein et al., 1997), the DMB-derivatized carbohydrates were chromatographed on a TosoHaas ODS-120T column (25 cm×2.1 mm) and eluted with a 40 min gradient of 7 % methanol, 0.1 % acetic acid to 7 % methanol, 0.1 % acetic acid, 20 % acetonitrile and injected into a Hewlett-Packard (Palo Alto, CA, USA) 1100MSD single quad mass spectrometer. Typical positive and negative electrospray ionization conditions were used, and the scan range was set from 120 to 1200 atomic mass units. Fragmentation of the analyte ions was accomplished by increasing the differential voltage between the capillary and second skimmer. To control for the efficient detection of sialic acids and related sugars, pure NANA, KDN and KDO were derivatized, chromatographed and injected into the mass spectrometer in the same fashion.

Results

Specific adhesion of ookinetes to the lumen side of mosquito midguts

Midgut halves, termed gut sheets, were isolated from blood-fed mosquitoes. Each gut sheet has an area of approximately 0.5 mm^2 and contains approximately 10^4 midgut cells. The gut sheets used in this study were completely free of blood cells, as judged by light and electron microscopy (Zieler et al., 1998). Each gut sheet has two distinct surfaces: one is covered with microvilli and is in contact with the peritrophic matrix and blood meal in the gut lumen after blood-feeding; the other is covered by a basement membrane, is lined with tracheoles and muscle fibres and is in contact with the insect haemolymph (Hecker, 1977). These two sides will be referred to as the lumen side and haemolymph side of the midgut, respectively.

Purified *Plasmodium gallinaceum* ookinetes bind to isolated mosquito midguts when parasites and midguts are combined in a tube and the parasites are allowed to settle on the midgut sheets. However, the binding is enhanced when mild centrifugation is used to bring the ookinetes into contact with the midguts (Shahabuddin and Pimenta, 1998). Fluorescently labelled ookinetes bound to gut sheets were fixed and examined under a fluorescence microscope to detect and quantify bound ookinetes. The haemolymph side of the gut sheet can be distinguished by focusing on the tracheoles, which are attached to the basement membrane (Fig. 1B). Thus, we were able to determine to which side a particular ookinete was bound.

To determine the specificity of ookinete binding to the midgut sheets, we examined the interactions of the midguts and ookinetes with a variety of tissues, cell types and other substrates. We tested whether ookinetes have a general ability to bind to different mosquito tissues and insect cell types. First, the relative levels of ookinete binding to the lumen and

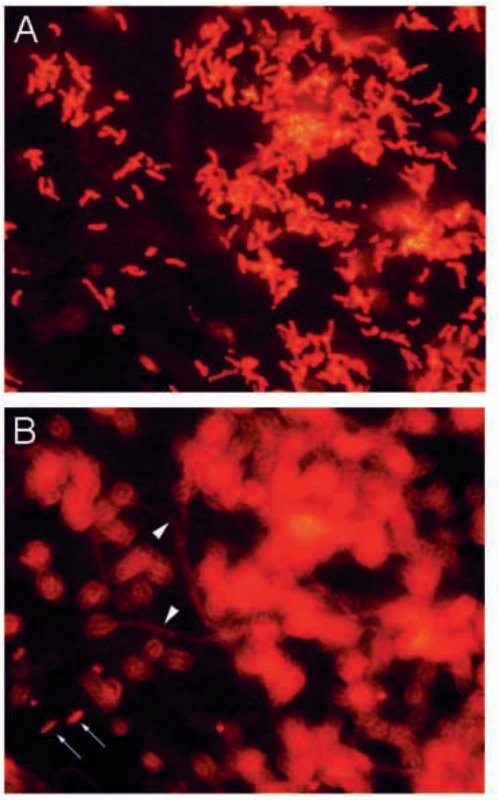

Fig. 1. *Plasmodium gallinaceum* ookinetes bound to midgut sheets. The ookinetes are stained red with the fluorescent membrane dye PKH26. (A) Ookinetes bound to the lumen side of the midgut. (B) Few ookinetes bound to the haemolymph side of the mosquito midgut (arrows). The remaining fluorescence comes from out-of-focus ookinetes bound to the lumen side of the midgut. Tracheoles found on the haemolymph side can be seen in focus (arrowheads). 200×.

haemolymph sides of the midgut sheets were quantified. Averaged over 12 experiments, each with a different preparation of ookinetes and midguts, 94% of the bound ookinetes were found on the lumen surface, the rest being on the haemolymph side (see Fig. 1). The adhesion of ookinetes to ovaries, Malpighian tubules and the *Drosophila melanogaster* S2 cell line was also tested. Almost no binding of ookinetes to these tissues was observed (data not shown). We conclude that there is a specific ligand on the lumen side of mosquito midguts to which ookinetes bind with high preference.

We next examined whether mosquito midguts have general adhesive properties and found that untransformed *P. gallinaceum* zygotes, macrogametes and mammalian cells did not bind to the lumen side of midgut sheets. In our parasite preparations, ookinetes are present as a mixture with untransformed zygotes and macrogametes and account for 20–80% of the total parasites, depending on the preparation. However, after binding to midgut sheets, 95% of the parasites bound to the lumen side are ookinetes, regardless of their initial proportion in the preparation. Table 1 summarizes three such experiments. We also attempted to bind a human B-lymphoblastoid cell line, UC729-6, to isolated midgut sheets. The UC729-6 cells completely failed to adhere to midguts. We conclude that ookinetes have a specific property which allows them to bind to the lumen surface of the mosquito midgut with high affinity.

We further investigated some of the non-specific binding properties of ookinetes. Ookinetes are known to adhere to a variety of surfaces, including glass slides (Freyvogel, 1966; Shahabuddin et al., 1997). To determine whether ookinete binding to glass and to the midgut lumen have similar properties, we performed adhesion experiments in the presence of different proteins and glycoproteins, which were added as non-specific competitors. The four proteins (BSA, fetuin, orosomucoid and ovalbumin) were chosen for their varied glycosylation patterns. As shown in Fig. 2A, the binding of ookinetes to the lumen side of midgut sheets is not affected by the presence of any of the four proteins. In contrast, their binding to glass slides was inhibited significantly at protein concentrations much lower than those used for the midgut adhesion assays (Fig. 2B). These data further support our conclusion that ookinete binding to mosquito midguts is specific, while their interactions with other surfaces are not.

Ookinetes seem to bind equally well to midgut sheets derived from unfed and blood-fed mosquitoes. When binding to midgut sheets from unfed mosquitoes, they also show a strong preference for the lumen side (data not shown). These

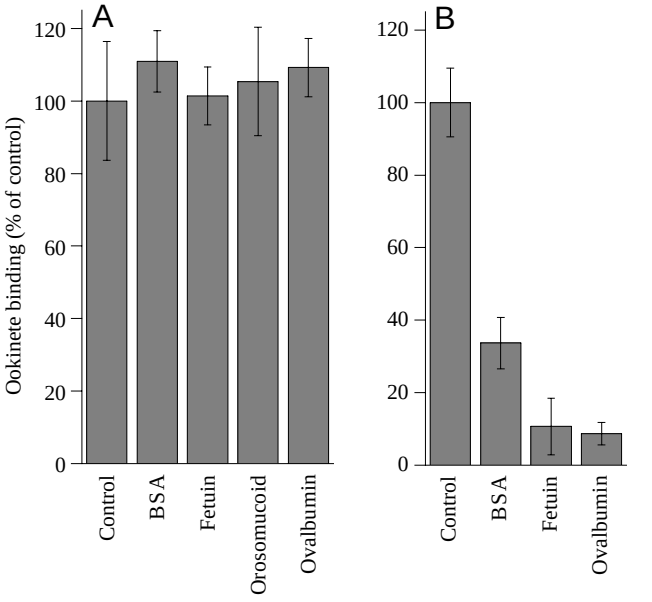

Fig. 2. Ookinetes bind specifically to the lumen surface of the mosquito midgut. (A) Various proteins added to the adhesion reaction at a final concentration of 20 mg ml^{-1} fail to inhibit ookinete binding. (B) Three of the same proteins at a final concentration of 6 mg ml^{-1} inhibit ookinete binding to glass slides. The inhibition by all three proteins is statistically significant (BSA P=0.0018; fetuin P=0.0001; ovalbumin P<0.0001). Orosomucoid was not tested for inhibition of ookinete binding to glass slides. Control: no protein was added to the adhesion reaction mixture. All error bars represent standard errors of the mean (N=4). BSA, bovine serum albumin.

Table 1. *Relative binding of ookinetes and zygotes to the lumen side of mosquito midguts*

Experiment number	% ookinetes[a]/ % zygotes[b] in preparation	Total number of cells bound	Bound ookinetes[a] (%)	Bound zygotes[b] (%)
1	24/76	1376	95	5
2	48/52	2158	93	7
3	56/44	2120	96	4
Total	45/55	5654	95	5

[a]Ookinetes are distinguished from other cell types by their elongate shape.
[b]Zygotes in this case refers to any cell that is round in shape; round cells found in our preparations include untransformed zygotes and unfertilized macrogametes.

observations suggest that the adhesion ligand is already present on the midgut surface before blood feeding. However, only blood-fed midgut sheets were used in the quantitative adhesion experiments described in this study.

The midgut ligand for ookinetes is a carbohydrate

To determine whether midgut-associated carbohydrates are necessary for ookinete binding, exposed carbohydrates were destroyed by pre-treatment of the midguts with various concentrations of sodium meta-periodate. The capacity of the midguts to bind ookinetes was effectively destroyed by sodium meta-periodate in a concentration-dependent fashion. The lumen side of the mosquito midgut appears to be very sensitive to low periodate concentrations, since 1 mmol l^{-1} periodate at pH 5.5 is sufficient to destroy adhesion completely (Fig. 3A). Concentrations of periodate below 1 mmol l^{-1} result in intermediate levels of binding. This finding suggests that the midgut ligand for ookinetes is a carbohydrate with a high degree of periodate-sensitivity.

To examine the characteristics of the midgut ligand, various monosaccharides were tested for their ability to compete with ookinete binding to mosquito midguts. The sugars tested were glucose, *N*-acetylglucosamine, galactose, *N*-acetylgalactosamine, *N*-acetylneuraminic acid (NANA), mannose and fucose. All the sugars were added to the adhesion reaction at final concentrations of 20 mmol l^{-1}. NANA was the

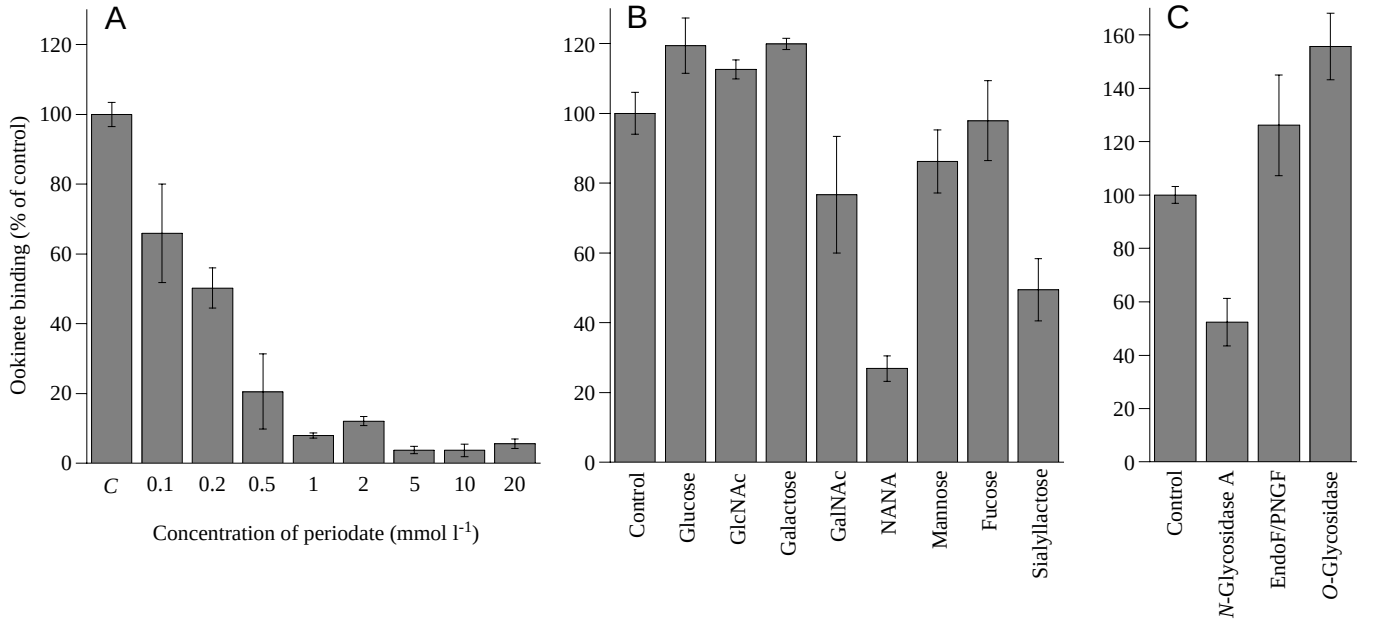

Fig. 3. Ookinetes bind to carbohydates on the lumen surface of mosquito midguts. (A) Periodate treatment of midgut sheets results in loss of ookinete binding. Midgut sheets were treated with various concentrations of sodium periodate for 30 min, washed and exposed to ookinetes. The effect of periodate treatment on ookinete binding is statistically significant at all concentrations of periodate tested (0.1 mmol l^{-1} P=0.014; all other concentrations P<0.0001). *C*, control, buffer only. (B) Inhibition of ookinete binding by monosaccharides. The sugars were added to adhesion reactions at a final concentration of 20 mmol l^{-1} except for sialyllactose, which was present at 13 mmol l^{-1}. *P*-values of the *t*-test comparing monosaccharide results with the control group were: glucose P=0.10; *N*-acetylglucosamine (GlcNAc) P=0.10; galactose P=0.019; *N*-acetylgalactosamine (GalNAc) P=0.24; *N*-acetylneuraminic acid (NANA) P<0.0001; mannose P=0.25; fucose P=1.0; sialyllactose P=0.003. (C) Reduction of ookinete binding by pre-treatment of the midguts with *N*-glycosidase A. Other *N*- or *O*-glycosidases do not reduce binding. The inhibition by *N*-glycosidase A is significant (P<0.0001). All error bars represent standard errors of the mean (N=4–10). EndoF/PNGF, EndoF/Peptide-*N*-glycosidase F.

only monosaccharide that blocked ookinete adhesion by more than 50 % (Fig. 3B). Sialyllactose, a mixture of α(2,3)- and α(2,6)-linked isomers, also inhibited the binding significantly. Smaller effects were observed with *N*-acetylgalactosamine and mannose; however, these effects were not statistically significant (see legend to Fig. 3 for *P* values).

To test whether the carbohydrate ligand is linked to glycoproteins, midgut sheets were treated with a variety of general glycosidases. Treatment of the gut sheets with *N*-glycosidase A significantly reduced binding (Fig. 3C; see the figure legend for statistical values). A mixture of Endo-F and Peptide-*N*-glycosidase F had no effect, nor was any effect seen with an *O*-glycosidase (endo-α-*N*-acetylgalactosaminidase).

The degree of ookinete adhesion to the midgut lumen correlates with levels of oocyst formation

To determine whether ookinete adhesion to the midgut epithelium plays a role in the ability of the ookinete to invade the midgut and form oocysts within the mosquito, we measured adhesion levels of ookinetes *in vitro* and the intensity of oocyst formation *in vivo* after feeding ookinete-containing blood to mosquitoes. To enable a correlation between these two processes, we screened a variety of reagents for their ability to inhibit ookinete adhesion *in vitro* and to be readily ingested by mosquitoes as part of a blood meal. Two reagents were chosen: a total carbohydrate extract prepared from adult *Aedes aegypti* and H.46, a horse polyclonal antiserum raised against the K1 strain of *E. coli*, which is reactive with polysialic acids and other bacterial carbohydrates (Troy et al., 1987). Either reagent was mixed into chicken blood together with ookinetes and fed to female *Aedes aegypti*. This technique bypasses the pre-ookinete stages of parasite development and ensures that any effects of the reagents on oocyst formation must be due to inhibition at the ookinete stage or later. Midguts were removed from the mosquitoes 8 days later and examined for oocysts; oocyst numbers were compared between the test groups and mosquitoes that were fed control reagents. As shown in Table 2, both reagents inhibit adhesion and oocyst formation at similar levels. The carbohydrate extract reduces adhesion *in vitro* by more than 70 %; oocyst levels are 88 % lower in all mosquitoes, and 57 % lower in mosquitoes with at least one oocyst (see Table 2). The H.46 antiserum inhibits adhesion *in vitro* by 75 %; mean oocyst numbers are reduced by 68 % compared with the control value among all dissected mosquitoes and by 49 % among mosquitoes with at least one oocyst (Table 2).

To exclude the possibility that the reagents directly affect ookinete viability or permanently alter their normal functioning, each reagent was added to an ookinete suspension and incubated for 2 h. The ookinetes were then washed, resuspended in chicken blood and fed to *Aedes aegypti* mosquitoes. No difference in oocyst numbers was observed between mosquitoes fed treated ookinetes and those fed untreated ones (data not shown). Therefore, the reagents do not act by reducing ookinete viability and must be present in the blood meal to inhibit oocyst formation. A small sample of the same ookinetes was also stained with ethidium homodimer, a dye that specifically stains dead cells (Shahabuddin et al., 1998). There was no difference between treated and untreated ookinetes in the percentage of cells stained with ethidium homodimer (data not shown).

Table 2. *Correlation between ookinete–midgut adhesion and oocyst formation*

	Ookinete-midgut adhesion		Oocyst formation			
	Ookinetes bound per midgut[a]	*P*[b]	Infected mosquitoes/ total[c]	Mean oocyst numbers[d]	Intensity of infection[e]	*P*[f]
Mosquito carbohydrate[g]	28±5	<0.0001	8/26	0.5 (0–10)	2.5 (1–10)	<0.0001
Buffer only	100±2		21/25	4.1 (0–91)	5.8 (1–91)	
H.46 horse antiserum[h]	25±5	<0.0001	18/26	2.7 (0–42)	4.5 (1–42)	0.003
Control horse serum[k]	100±5		24/25	8.5 (0–296)	8.8 (1–296)	

[a]Ookinete adhesion is represented as the percentage of mean ookinete numbers adhering to one midgut relative to the control group. The S.E.M. of ookinete numbers from eight different measurements (mosquito carbohydrate) or 14 measurements (H.46 antiserum) is shown.

[b]*P* values were determined using the *t*-test, comparing the relative number of ookinetes per midgut in each group with the control group.

[c]Number of mosquitoes infected with oocysts/total number of gravid mosquitoes dissected.

[d]Oocyst numbers are represented as the geometric mean with ranges in parentheses.

[e]Geometric mean of oocysts among midguts with at least one oocyst; ranges are in parentheses.

[f]*P* values were determined using Mann–Whitney rank sum analysis, comparing the number of oocysts in each group with the control group.

[g]The final concentration of total mosquito carbohydrate was approximately 7 mg ml^{-1} in all experiments.

[h]The H.46 serum was diluted 1:10 in all experiments.

[k]A commercially available horse serum was used as a control at a 1:10 dilution in all experiments.

No sialic acids are present in the mosquito midgut

The results obtained from periodate treatment of midguts and competition for ookinete binding by *N*-acetylneuraminic acid suggest that ookinetes may bind to a sialic-acid-like carbohydrate on the midgut surface. Since sialic acids are generally not thought to be present on insect glycoproteins or glycolipids (Maerz et al., 1995), we performed an exhaustive search for sialic acids in mosquito midguts. A highly sensitive sialic acid assay was used to analyze the contents of acid hydrolysates derived from 500–1000 homogenized midguts from unfed mosquitoes (Stanton et al., 1995; Klein et al., 1997).

The DMB assay used in this study is based on derivatization of the sialic acid monomers liberated by acid hydrolysis with 1,2-diamino-4,5-methylenedioxybenzene (DMB) (Hara et al., 1986, 1987). The resulting fluorescent compounds were separated by reversed-phase HPLC and were characterized structurally using an in-line electrospray mass spectrometer. This method is able to detect sialic acids with any type of modification, as well as the related deamino sugars KDO and KDN (Klein et al., 1997). An exhaustive examination of all the fluorescent peaks obtained from a variety of midgut hydrolysates revealed no compounds with molecular masses characteristic of sialic acids or sialic acid derivatives (data not shown). Furthermore, at higher differential voltages, none of the ions present in the fluorescent peaks showed fragmentation patterns characteristic of sialic acids (Klein et al., 1997). We conclude that no sialic acids are present in the midgut of the unfed mosquito.

Discussion

In this study, we have characterized one of the early interactions between *Plasmodium gallinaceum* ookinetes and mosquito tissues during the complex journey of the ookinete from the interior of the insect midgut to the basement membrane surrounding the midgut. Genetically and morphologically, *P. gallinaceum* is closely related to *P. falciparum*, the most widespread and pathogenic of the human malaria parasites (Waters et al., 1991; McCutchan et al., 1996). Both parasites express related antigens on the surface of their zygotes and ookinetes (Kaslow et al., 1989; Duffy et al., 1993). However, no method exists for preparing ookinetes of *P. falciparum* and we have therefore used *Plasmodium gallinaceum* as a model system.

Our *ex vivo* system of interacting, in an Eppendorf tube, purified ookinetes with midgut epithelia dissected from blood-fed mosquitoes (Shahabuddin et al., 1997; Shahabuddin and Pimenta, 1998) has provided us with a new tool to approach the important questions about the migration of the ookinete on and through the midgut epithelium. Very simple manipulations allow us to bind ookinetes to midgut sheets and to investigate the specificity and molecular nature of this adhesive interaction. Our data suggest that ookinetes interact specifically with the lumen side of the midgut epithelium. We postulate that there is a ligand on the midgut surface to which the ookinete binds as one of its first recognition events after emerging from the blood meal. A strong correlation exists between the efficiency of ookinete adhesion *in vitro* and oocyst formation in the mosquito. Therefore, the adhesion of ookinetes to mosquito midguts *in vitro* probably reflects an interaction that occurs during the migration of the parasite through the mosquito and which is necessary for the ookinete to find its ultimate target, namely the basement membrane of the midgut.

We have observed in a separate study that, when ookinetes are bound to mosquito midgut sheets and are given time to invade, only a small proportion of the bound parasites will invade the epithelium. The ookinetes appear to invade a particular cell type, which occurs relatively infrequently in the midgut, accounting for only a small percentage of all midgut cells (Shahabuddin and Pimenta, 1998). We think that the initial binding of the ookinete to the midgut surface described in the present study, although essential, is independent of the recognition and invasion of the appropriate cells. Therefore, we propose a two-stage model of ookinete–midgut interactions (Fig. 4). After leaving the blood meal (Fig. 4, part 1) and penetrating through the peritrophic membrane (Fig. 4, part 2), ookinetes contact the midgut surface to which they adhere by recognizing a specific carbohydrate receptor (Fig. 4, part 3). This is the first stage of the interaction between the parasite and the midgut epithelium. Staying close to the luminal midgut surface, the parasites now move around two-dimensionally on the surface of the epithelium in search of invasion sites (Fig. 4, part 4). Once the correct cell type is located, other recognition steps allow the parasites to arrest their surface migration and to invade the epithelium at this site (Fig. 4, part 5). Invasion constitutes the second stage of interaction. The purpose of the intial binding in Fig. 4 part 3 is therefore to increase the chance of finding the appropriate cells that are permissive to invasion.

Ookinete adhesion is abolished when the mosquito midgut

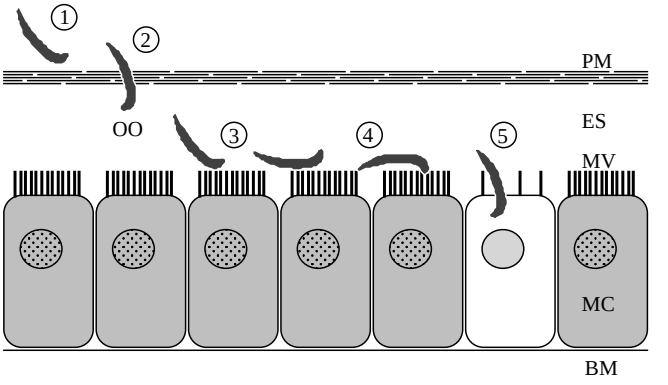

Fig. 4. Biphasic model of ookinete adhesion to the midgut epithelium and invasion of morphologically distinct midgut cells. After emerging from the blood meal (1), the ookinete binds to the peritrophic membrane (PM) and penetrates through it (2). It then crosses the ectoperitrophic space (ES) and adheres to the midgut epithelium by interacting with a midgut ligand (3). The parasite then migrates over the surface of the epithelium in search of a suitable invasion site (4). When the ookinete encounters a cell type permissive for invasion, it arrests its migration and invades the cell (5). BM, basement membrane; MC, midgut cell; MV, microvilli; OO, ookinete.

is pre-treated with sodium periodate, which selectively oxidizes sugar groups with vicinal hydroxyl groups. This finding strongly suggests that the ookinete binds to a midgut carbohydrate ligand. Ookinete adhesion is also reduced in the presence of total carbohydrates extracted from adult mosquitoes, suggesting competition for binding sites (Table 2). Furthermore, ookinete binding is reduced when the midguts are pre-treated with *N*-glycosidase A, which is a general *N*-glycosidase that hydrolyzes all types of *N*-glycans, even those with the α1,3-bound core fucose residues that are common in insect glycoproteins (Plummer et al., 1987; Tretter et al., 1991, 1993). Other general *N*-glycosidases (PNGase F, peptide *N*-glycosidase F) are sensitive to core fucosylation (Tretter et al., 1991) and do not reduce binding. As a result, we consider it likely that ookinetes bind to glycoproteins on the midgut surface that have a specific type of *N*-linked glycan unique to the midgut lumen and not found on the basement membranes of the midgut or other mosquito tissues.

Two additional observations caused us to narrow our search for the carbohydrate ligand. The sensitivity of the midgut ligand to low concentrations of periodate (1 mmol l^{-1} and below) suggested a sialic-acid-like sugar. It has long been recognized that sialic acids, of all carbohydrates, are the most rapidly oxidized by sodium periodate (McLean et al., 1971; Van Lenten and Ashwell, 1971; Reuter et al., 1989), and this feature is often exploited to allow selective oxidation of sialic acids in complex mixtures of carbohydrates (Suttajit and Winzler, 1971; Veh et al., 1976; Powell et al., 1993; Sgroi et al., 1993). Furthermore, we found that free *N*-acetylneuraminic acid competed with ookinete binding to midguts.

The presence of sialic acids as authentic constituents of adult insect cells is still controversial (Maerz et al., 1995; Altmann, 1996). Several studies of glycoproteins purified from insect cell lines (derived from embryonal insect tissues) have failed to provide any evidence for sialylation (Thomsen et al., 1990; Luo et al., 1992; Voss et al., 1993; Kulakosky et al., 1998). Other studies did detect sialic acids in insect cell lines (Davidson et al., 1990; Davidson and Castellino, 1991; Davis and Wood, 1995), although in one case a more rigorous analysis refuted the earlier finding (Kulakosky et al., 1998). There is one published report of the detection of sialic acids in early *Drosophila melanogaster* embryos using rigorous techniques (Roth et al., 1992). We used a variety of acid hydrolytic conditions in combination with a very sensitive and specific sialic acid assay (derivatization with DMB followed by HPLC and fluorescent detection, and electrospray mass spectrometric analysis of the fluorescent peaks) to show that unfed *Aedes aegypti* midguts do not contain any sialic acids. Since large numbers of midguts (500–1000) were used in each detection attempt, representing approximately 10^7 to 2×10^7 midgut cells, we consider it unlikely that our failure to detect sialic acids can be attributed to a lack of starting material. We propose that a novel, possibly acidic, sugar that shares at least one of the properties of sialic acids, sensitivity to low concentrations of sodium periodate, may be present in mosquito midgut.

The long-term aim of this work is to understand the interactions between the ookinete and the mosquito midgut, and to identify target insect molecules that can be changed in the mosquito to block the development and transmission of the parasite. The midgut is the earliest interface between the insect and the parasite and is generally considered to be the best candidate tissue for disrupting the malarial life cycle within the mosquito. On the basis of the findings of our studies, it may be possible to design new malaria control strategies in the future by modifying vector mosquitoes to make them refractory to malaria transmission (Gwadz, 1994; Collins and Paskewitz, 1995).

We thank Drs Louis H. Miller, José M.C. Ribeiro and Lewis K. Pannell for their support and encouragement during this work, for numerous helpful discussions and for their comments on the manuscripts. Thanks especially to José for his help with setting up the HPLC assays for sialic acid detection. The authors are also greatly indebted to Drs David D. Roberts, Willie F. Vann and Gilbert Ashwell for their advice and suggestions on carbohydrates and glycobiology. Special thanks to Gil Ashwell for his help preparing the mosquito carbohydrate extract, trying to detect sialic acids and for his time discussing our work. We are also grateful to Willie Vann for his gift of a lifetime supply of the H.46 antiserum. We would further like to thank Drs David C. Kaslow, Jesus Valenzuela, Joseph Vinetz, Thomas Templeton, Stephane Cociancich and David Keister for their support and for frequent interesting discussions. This work was partly supported by the WHO/TDR grant 950476 to Louis Miller. H.Z. is supported by the Vector Biology Network of the John D. and Catherine T. MacArthur Foundation.

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