Identification of Novel *Plasmodium gallinaceum* Zygote- and Ookinete-Expressed Proteins as Targets for Blocking Malaria Transmission

Rebecca C. Langer,[†] Fengwu Li, and Joseph M. Vinetz*

WHO Collaborating Center for Tropical Disease, Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555

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The development of transmission-blocking vaccines is one approach to malaria control. To identify novel *Plasmodium* zygote- and ookinete-secreted proteins as targets of blocking malaria transmission, monoclonal antibodies (MAbs) were produced against parasite-secreted proteins found in *Plasmodium gallinaceum* ookinete culture supernatants. Four MAbs—1A6, 2A5, 2B5, and 4B6—were identified that bound to *P. gallinaceum* zygotes and ookinetes in diverse patterns in terms of spatial localization on parasites, time course of antigen expression, and Western immunoblot patterns. MAbs 2A5 and 4B6 recognized more than one protein band as detected by Western immunoblot of *P. gallinaceum* ookinete supernatants. Beginning at 0 h postfertilization, MAb 2A5 recognized a diverse set of antigens; at 10 h postfertilization, MAb 4B6 recognized several antigens as well. MAb 1A6 recognized a single ~17-kDa protein, and 2B5 recognized a single ~32-kDa protein at 15 h postfertilization. In membrane feeding assays to assess the effect of these MAbs on *P. gallinaceum* infectivity for *Aedes aegypti* mosquitoes, the addition of MAbs 1A6 and 2B5 to infectious blood meals significantly inhibited oocyst development in the mosquito midgut. In contrast, MAb 2A5 seemed to enhance infectivity. These results demonstrate that *Plasmodium* ookinetes secrete proteins (in addition to previously characterized chitinases) that may be targets for blocking malaria transmission. Future investigation of ookinete-secreted neutralization-sensitive molecules should provide valuable insight into mechanisms by which ookinetes exit the blood meal, penetrate and transverse the peritrophic matrix, and invade the mosquito midgut epithelium.

One strategy to achieve global malaria control includes the development of transmission blocking vaccines. This approach targets antigens expressed by *Plasmodium* parasites either on gametocytes within the human host or within the mosquito (3). Antigens expressed by *Plasmodium* stages within the mosquito midgut are logical to consider for transmission blocking vaccines since the mosquito midgut stages of the malaria parasite represent a genetic bottleneck: many asexual-stage parasites in the vertebrate host are winnowed down by orders of magnitude to a few ookinetes ending up as oocysts on the mosquito midgut epithelium. Studies over the past two decades have begun to elucidate *Plasmodium* ookinete molecules that are necessary for invasion of the mosquito midgut. Secreted ookinete antigens may be useful as synergistic components of transmission blocking vaccines since molecules sequentially secreted from secretory organelles (micronemes, rhoptries, and dense granules) in apicomplexan parasites play a critical role in target cell invasion (2, 4, 6, 13, 17, 18). Few *Plasmodium* ookinete-secreted antigens have been characterized, despite numerous molecules being secreted at this stage (as determined by Coomassie blue staining of ookinete culture supernatants; R. C. Langer and J. M. Vinetz, unpublished data). *Plasmodium*

ookinete-secreted chitinases have been shown to be necessary for the ookinete to exit the blood meal and transverse the peritrophic matrix (5, 20, 21, 23, 24). Characterization of *Plasmodium* chitinases has demonstrated that ookinete-secreted molecules are targets of blocking parasite invasion of the mosquito midgut. Inhibition of chitinase activity with either chitinase antagonists, such as allosamidin (20), or *Plasmodium* chitinase-specific antibodies (R. C. Langer, F. Li, and J. M. Vinetz, unpublished data) significantly reduces the number of oocysts developing in the mosquito midguts. A successful *Plasmodium* transmission blocking vaccine will likely target numerous zygote and ookinete molecules, both surface-associated and soluble secreted proteins, such as the 25- and 28-kDa families of sexual-stage surface proteins (7, 8, 10, 22). To dissect further molecular mechanisms by which *Plasmodium* ookinetes invade the mosquito midgut and simultaneously identify potential transmission-blocking vaccine candidates, we produced a panel of monoclonal antibodies (MAbs) against soluble proteins found in supernatants of *Plasmodium gallinaceum* ookinetes. Because large numbers of ookinetes can be cultured in vitro in chemically defined, axenic medium for the avian malaria parasite, *P. gallinaceum* (11), our studies have focused on that species. *P. gallinaceum* is closely related to the human malaria parasite, *Plasmodium falciparum* (15, 16, 25). Delineation of antigens secreted by the avian parasite will likely lead to the identification of homologs in the *P. falciparum* genome database, which would be an attempt to define one component of the proteome of the *P. falciparum* ookinete which, to date, has not been cultivatable in vitro.

* Corresponding author. Mailing address: WHO Collaborating Center for Tropical Disease, Keiller 2.138, University of Texas Medical Branch, Galveston, TX 77555-0609. Phone: (409) 747-2962. Fax: (409) 747-2437. E-mail: jovinetz@utmb.edu.

[†] Present address: University of Texas School of Public Health, Houston, TX 77030.

MATERIALS AND METHODS

Production of *P. gallinaceum* ookinete culture supernatants. The 8A strain of *P. gallinaceum* was used to infect 4- to 6-week-old White Leghorn chickens. A gametocyte-producing line was maintained by subpassage in chickens and periodic passage through mosquitoes. Ookinetes were cultured from purified zygotes in serum-free and protease-free M199 culture medium as described previously (11). Twenty-four- to thirty-hour cultures of ookinetes were centrifuged ($4,000 \times g$, 10 min), parasites were pelleted, and culture supernatants were collected and stored at -20°C until use. Pooled supernatants were concentrated 60-fold by centrifugal ultrafiltration (Centriprep 10; Amicon).

Production of MAbs against *P. gallinaceum* ookinete culture supernatant antigens. *P. gallinaceum* ookinete culture supernatants were prepared as described above and protein concentration determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.). Chitinase activity present in ookinete culture supernatants was confirmed by a microfluorimeter assay using the substrate 4-methylumbelliferyl-*N,N'*- β -D-triacetylchitotrioside (Calbiochem, La Jolla, Calif.) as previously described (24), confirming the presence of known ookinete-secreted proteins (chitinases) at biologically detectable concentrations.

Eight-week-old BALB/c mice (Charles River Laboratories) were immunized intraperitoneally with *P. gallinaceum* ookinete culture supernatant emulsified in complete Freund adjuvant (Difco, Detroit, Mich.) and boosted (50 $\mu\text{g}/\text{mouse}$ in incomplete Freund adjuvant) by the same routes 2, 6, and 10 weeks later. At 4 weeks after the last boost, a final injection was administered (50 $\mu\text{g}/\text{mouse}$ intraperitoneally and 50 $\mu\text{g}/\text{mouse}$ intravenously). Spleen cells were fused 3 days later with SP2/0 myeloma cells as previously described (9). Hybridomas were screened by enzyme-linked immunosorbent assay for immunoreactivity against concentrated *P. gallinaceum* supernatants. Positive hybridomas were cloned by single cell limiting dilution in Dulbecco modified Eagle medium containing 1% penicillin-streptomycin and 10% fetal bovine serum (Omega Scientific, Tarzana, Calif.), and the isotype was determined by using a mouse antibody isotyping kit (Gibco, Grand Island, N.Y.). Hybridoma culture supernatants were collected for each clone (1A6, 2A5, 2B5, and 4B6), and the MAb concentration was determined by radial immunodiffusion (Binding Site, San Diego, Calif.).

Characterization of anti-*P. gallinaceum* ookinete supernatant MAbs by immunofluorescence microscopy and Western immunoblotting. Aliquots were taken from in vitro ookinete cultures at 0, 10, 15, and 20 h postexflagellation and fertilization (hpf), centrifuged (10 min, $3,000 \times g$, 22°C) and parasite pellets suspended in phosphate-buffered saline (PBS) containing 3% (wt/vol) bovine serum albumin. The parasite preparations were divided into aliquots (20 $\mu\text{l}/\text{well}$) on multichamber glass slides (PGC Scientific, Frederick, Md.). Slides were allowed to air dry and were then heat fixed. Ookinete slides were incubated (1 h, 22°C) with PBS-3% bovine serum albumin-3% (vol/vol) Triton X-100 to block nonspecific binding sites and permeabilize parasites. Slides were incubated (30 min, 22°C) in a humidified chamber with primary MAbs (including isotype-matched negative controls), washed extensively with PBS, incubated with affinity-purified fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG)/M/A (Kirkegaard & Perry, Gaithersburg, N.J.), and washed with PBS, followed by a final H_2O wash. Slides were mounted with Permafluor (Immunon Shandon, Pittsburgh, Pa.) and observed and photographed on a Zeiss Axiophot 2 immunofluorescence microscope.

For Western immunoblotting (nonreducing, denaturing), *P. gallinaceum* ookinete culture supernatants collected at 0, 10, 15, and 20 hpf (50 μg) were heated (5 min, 90°C) in sample buffer (25 mM Tris, pH 6.8; 2.2% [wt/vol] sodium dodecyl sulfate; 15% [vol/vol] glycerol; 0.001% [wt/vol] bromophenol blue), centrifuged ($10,000 \times g$, 5 min) to remove insoluble debris and resolved in 4 to 20% Tris-glycine gradient gels (Invitrogen, Carlsbad, Calif.). Resolved proteins were electroblotted to nitrocellulose membranes by using the Novex Xcell Blot II module. After blocking with Buffer A (0.17 M NaCl, 0.1 M Tris [pH 7.5]) containing 5% (wt/vol) nonfat dry milk (NDM), blots were incubated (22°C , 1 h) with primary antibodies in Buffer B (Buffer A containing 0.1% [wt/vol] sodium dodecyl sulfate and 0.1% [vol/vol] Triton X-100) containing 5% (wt/vol) NDM. Primary MAbs included IgG1 isotype control of irrelevant specificity, IgM isotype control of irrelevant specificity, and the MAbs 1A6, 2A5, 2B5, and 4B6 ($\sim 25 \mu\text{g}$ of each/ml). After six washes (5 min each) with Buffer B containing NDM, blots were incubated (22°C , 1 h) with alkaline phosphatase-conjugated goat anti-mouse IgG/M/A (Kirkegaard & Perry) diluted 1:2,000 in Buffer B containing NDM. Blots were washed three times with Buffer B containing NDM, three times with Buffer B only, and one time with 0.1 M Tris (pH 9.0) and then developed with BCIP/NBT alkaline phosphatase substrate (Kirkegaard & Perry).

Membrane feeding assay. Mosquitoes were fed blood meals through a membrane as previously described (19), with the exception that chicken skin was used in place of parafilm. Allosamidin (10 $\mu\text{g}/\text{ml}$), a potent chitinase inhibitor used as

a positive control in these experiments (20), or anti-*P. gallinaceum* supernatant or isotype control MAbs (10 $\mu\text{g}/\text{ml}$) were mixed with freshly drawn *P. gallinaceum*-infected ($\sim 10\%$ parasitemia) chicken blood (200 μl) containing heparin and fed (20 min, 37°C) to overnight-starved *Aedes aegypti* mosquitoes. To assess the transmission blocking activity of the MAbs, mosquitoes were dissected at 7 days post-blood meal, midguts stained with 1% (vol/vol) mercurochrome in water, and the number of oocysts per midgut was enumerated under light microscopy. Oocyst numbers in experimental and control groups were compared statistically by the Mann-Whitney U test as previously described (8).

RESULTS AND DISCUSSION

Development of MAbs that recognize distinct, novel *P. gallinaceum* zygote-ookinete, time-dependent epitopes. Four stable hybridomas (1A6-IgG1, 2A5-IgG1, 2B5-IgG1, and 4B6-IgM) were identified to secrete MAbs that bound in distinct patterns to the surface, internal apical, and middle regions of ookinetes, zygotes, retorts, and extracellular (exogenous) antigen secreted by the parasites (Fig. 1), as determined by immunofluorescence microscopy. MAb 2A5 reacted with 100% of early zygotes (2.5 hpf and continued to react with 100% of the parasites through 24 hpf); this immunofluorescence pattern continued until full ookinete maturation, at which point the parasites appeared in clumps (Fig. 1A, 24 h). MAb 2A5 also bound in a pattern resembling cobwebs that seem to connect various parasites. MAb 4B6 reacted with $\sim 50\%$ of the early zygotes but 100% of the later stages. The majority of parasite-specific reactivity began 10 hpf on the surface of retorts, which progressed to the surface and apical tip of ookinetes at 15 and 24 hpf (Fig. 1B). MAb 4B6 recognized a remarkable amount of secreted antigen deposited on the slide at 15 and 24 hpf; this has been seen previously with ookinete-secreted chitinase (12). At time zero, few parasites were reactive with MAb 1A6, but at 10 and 15 hpf 100% of the parasites were strongly reactive. At 24 hpf, only $\sim 60\%$ of the parasites were immunoreactive with 1A6 (Fig. 1C). MAb 1A6 recognized structures resembling multiple pinpoint dots in the 24-hpf cultures; simultaneous light microscopy (not shown) showed that the 1A6-recognized antigen was localized on the extreme apical end of the ookinete. MAb 2B5 reacted with $<5\%$ of the parasites at time zero, with 100% at 10 and 15 hpf, and with $\sim 60\%$ of parasites 24 hpf. MAb 2B5 usually bound diffusely to zygotes and ookinetes but occasionally produced a granular pattern suggestive of subcellular organellar localization. Immunofluorescence microscopy with an IgG isotype-matched control showed insignificant background immunofluorescence (Fig. 1E).

Western immunoblot characterization of zygote or ookinete proteins recognized by MAbs. MAbs also reacted in distinct binding patterns with ookinete antigens derived from ookinete culture supernatants as determined by Western immunoblot (Fig. 2, antigen from 24 hpf culture supernatant shown). Both 2A5 and 4B6 recognized a range of nonreduced proteins. 1A6 recognized a single $\sim 17\text{-kDa}$ protein, and 2B5 recognized an $\sim 32\text{-kDa}$ protein; both of these proteins appeared most prominently 15 and 24 hpf but were also faintly visible at 10 hpf, as determined by the immunofluorescence microscopy approach described above.

Concentrations of proteins in ookinete culture supernatants may have been too low to detect minimal amounts in 10 hpf cultures by Western immunoblotting with the MAbs (data not shown), suggesting that these antigens had not been secreted in sufficient quantities for detection but remained in the apical

FIG. 1. Immunofluorescence microscopy of *P. gallinaceum* ookinete cultures collected at 0, 10, 15, and 24 h postfertilization. Immunofluorescence was performed with MAbs 2A5 (A), 4B6 (B), 1A6 (C), 2B5 (D), and an antigen-irrelevant IgG isotype control (E). Bar, 5 μ m.

organelles of the parasite detectable by immunofluorescence assay. The detection of immunoreactive antigens in the 24-hpf culture supernatants by Western immunoblot, despite diminishing reactivity by microscopic immunofluorescence assay, could indicate a higher concentration of the antigen in a secreted form as opposed to nonsecreted form.

Effect of MAbs on infectivity of *P. gallinaceum* for mosquitoes. Anti-*P. gallinaceum* ookinete supernatant MAbs were added to infectious blood meals at a concentration of 10 μ g/ml and fed to *Aedes aegypti* mosquitoes, and the development of oocysts in the mosquito midgut was enumerated. These mem-

brane feeding experiments were performed four times. MAbs 1A6 and 2B5 significantly reduced oocyst counts, with a geometric mean number of oocysts/gut somewhat less than the maximal effect produced by allosamidin (Table 1). The addition of similar quantities of irrelevant, isotype-matched control MAb had no effect on oocyst counts. Further molecular characterization and subcellular location of the two neutralization-sensitive antigens defined by MAbs 1A6 and 2B5 will be of substantial interest.

MAb 4B6 and isotype-matched control MAbs of irrelevant specificity had no significant effect on parasite development.

FIG. 2. Western immunoblot analysis of *P. gallinaceum* ookinete culture supernatants collected 24 h postfertilization. Molecular masses are indicated in kilodaltons at the left.

MAB 2A5 seemed to enhance oocyst development; the presence of this MAB in an infectious blood meal was associated with oocyst counts significantly above the negative control. It is possible that 2A5 recognizes an antigen targeted by a mosquito defense molecule or other similar enzymes and is efficiently serving as an inhibitor to a naturally occurring process. Additionally, 2A5 bound prominently to an ~36-kDa band often seen comigrating with an antigen that was immunoreactive with different IgG isotype control MABs of irrelevant specificities (data not shown). These results suggest the possibility of an IgG receptor expressed on *Plasmodium* ookinetes. If the parasites are expressing such a receptor that is tightly bound by a MAB specifically recognizing the receptor, the parasite may be protected from such events as host-mediated lysis. While these results do not indicate that 2A5 is a viable target of blocking transmission of malaria parasites, further characterization of the defined protein may provide detailed insight into ookinete interactions with the midgut and the response of the midgut to this process.

This study demonstrates that some of the numerous proteins secreted by *P. gallinaceum* ookinetes contain epitopes that may be new targets for blocking malaria transmission. None of the MABs described above produced Western immunoblot patterns resembling those produced by other known zygote or ookinete proteins. The proteins recognized by the transmission-blocking MABs—1A6 and 2B5—were primarily expressed 10 h or later, suggesting that these MABs are present and

biologically active relatively late after blood meal ingestion by the mosquito. In contrast, the leading transmission-blocking vaccine, the 25-kDa protein Pfs25, is expressed early in the mosquito midgut. Previous studies have also shown that MABs against Pfs25 and against mosquito salivary gland proteins persist long enough to be present in the oocyst, hemolymph, and even salivary gland (1, 14). Therefore, these data provide additional rationale for pursuing transmission-blocking vaccine candidates by targeting ookinete-specific proteins.

By creating a panel of MABs and screening for the ability to block transmission of the parasite to natural hosts, we have been able to begin the preliminary characterization of new zygote and ookinete proteins. More detailed analysis of ookinete-secreted antigens should provide valuable insight into mechanisms by which ookinetes invade the mosquito midgut. Future efforts will include focusing on molecular characterization of the transmission-blocking epitopes identified in this study and identification of their homologues in the *P. falciparum* genome database.

TABLE 1. Effect of MABs raised against *P. gallinaceum* ookinete-secreted proteins on oocyst development in *A. aegypti* mosquitoes

Treatment	No. of oocysts/gut ^a	Infectivity (%) ^b	No. infected/total ^c	P ^d
Expt 1				
2A5	7.17	208	34/40	0.001
4B6	4.63	135	31/37	NS
1A6	0.74	21.6	20/37	<0.001
2B5	0.49	14.2	12/40	<0.001
IgG-IgM IC ^e	3.44	100	34/40	
Allosamidin ^f	0.12	3.6	31/37	<0.001
Expt 2				
2A5	ND ^g	ND	ND	
4B6	2.11	57.4	29/40	NS
1A6	0.40	10.8	17/40	<0.001
2B5	1.30	35.4	21/40	0.002
IgG-IgM IC	3.69	100	29/36	
Allosamidin	0.05	1.2	2/40	<0.001
Expt 3				
2A5	52.0	137.4	37/40	0.009
4B6	33.65	88.9	38/40	NS
1A6	3.50	9.2	27/40	<0.001
2B5	7.64	20.2	24/40	0.001
IgG-IgM IC	37.83	100	39/40	
Allosamidin	0.07	0.2	3/40	<0.001
Expt 4				
2A5	57.88	155.4	39/39	0.011
4B6	36.54	98.1	40/40	NS
1A6	3.41	9.1	28/40	<0.001
2B5	12.37	33.2	36/40	<0.001
IgG-IgM IC	37.28	100	40/40	
Allosamidin	0.15	0.4	4/40	<0.001

^a Oocyst counts are presented as geometric means.

^b Infectivity is expressed as the percentage of geometric mean oocyst counts compared to the control group.

^c Number of mosquitoes infected/total number of mosquitoes dissected. Only mosquitoes with eggs, indicating blood meal ingestion, were dissected.

^d P values were determined by Mann-Whitney U test. NS, not significant.

^e IC, Isotype control MAB of irrelevant specificity.

^f Allosamidin, a chitinase inhibitor, was used as a positive control for transmission-blocking activity.

^g ND, not determined.

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