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A Surface Protein Expressed During the Transformation of Zygotes of *Plasmodium gallinaceum* is a Target of Transmission-Blocking Antibodies

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Antibodies against gametes of malarial parasites (*Plasmodium* spp.) have previously been shown to block infectivity of the parasites to mosquitoes by preventing fertilization of the parasites in the insect midgut. These antibodies did not have any effect on the development of fertilized parasites. We now report that a surface protein of M_r 26,000 synthesized by zygotes of *P. gallinaceum* is the target of antibodies which block infectivity of the fertilized parasites to mosquitoes. Identification of this target antigen offers a new stage of the parasite against which a malaria transmission-blocking vaccine could be developed.

Transmission of malaria from humans to mosquitoes is mediated by the sexual stages (gametocytes) of plasmodium formed as intraerythrocytic parasites in the blood of the host. Gametocytes undergo gametogenesis and fertilization in the midgut of a mosquito during a blood meal. During the next 24 h, the zygotes transform in the lumen of the midgut into ookinetes, invasive stages which penetrate the midgut wall and establish parasites in the mosquito tissues. Anti-gamete antibodies when ingested in a blood meal together with the parasites have been shown to block parasite fertilization and thus prevent infection in mosquitoes (1, 2). Species-specific monoclonal antibodies (MAbs) which blocked parasite fertilization in either the avian malaria *P. gallinaceum* (4) or the human malaria *P. falciparum* (8) systems recognized a set of three proteins of apparent M_r of ca. 55,000, 60,000 and 250,000 (depending upon parasite species) which are present on the surface of both male and female gametes of the parasites (2a). The fertilization-blocking antibodies had no effect, however, on the subsequent development of the parasites after fertilization was completed (4). We now report that a single MAb against a surface protein of M_r 26,000 synthesized during the transformation of zygotes of *P. gallinaceum* suppresses the development of the fertilized parasites in *Aedes aegypti* mosquitoes.

MATERIALS AND METHODS

Preparation of parasites, immune sera, and monoclonal antibodies. Preparations of purified male gametes (1) and ookinetes (4) of *P. gallinaceum* were made as previously described. Immune sera against each of these stages were raised in White Leghorn chicks, initially 5 weeks old, by three intravenous inoculations of 1×10^7 cells of the appropriate parasite preparation administered at 1-week intervals. Sera were collected 1 week after the last immunization and inactivated by heating at 56°C for 45 min. Hybridoma cell lines and MAbs were derived from a BALB/c mouse immunized with purified mature ookinetes of *P. gallinaceum* by methods previously described (4).

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Assay for effects of antibodies on infectivity of parasites to mosquitoes. The effects of the immune sera on the infectivity of parasites to *A. aegypti* mosquitoes were tested as previously described (4). The mosquitoes were fed through a membrane with *P. gallinaceum*-parasitized chicken blood cells suspended in the immune sera diluted in heat-inactivated normal chicken serum. Control mosquito batches were fed parasitized chicken blood suspended in normal heat-inactivated chicken serum without the addition of immune serum. The sera were mixed with the parasitized blood cells either before or after gametogenesis and fertilization were stimulated in vitro. In this way the effects of antibodies on the events leading to fertilization could be distinguished from those affecting the development of the fertilized zygotes.

When testing the effects of hybridoma-derived antibodies, the immune serum was replaced by heat-inactivated mouse ascitic fluid containing the appropriate antibody mixed in equal parts with heat-inactivated normal chicken serum.

Mosquitoes were dissected 1 week after membrane feeding, and their midguts were examined for the presence of oocysts. The number of oocysts (products of fertilization) per midgut was used as the measure of infectivity. For both fertilized and unfertilized parasites, the mean number of oocysts per gut in the control feeds was typically in the range of 50 to 100.

Labeling and identification of parasite proteins. The ookinetes were biosynthetically labeled by incubating 1×10^7 newly fertilized zygotes (2 to 3 h after gametogenesis) for 30 min at 22°C in 1 ml of RPMI 1640 medium (methionine and bicarbonate free) (GIBCO) with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Sigma) at pH 7.0 and containing 100 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.). The suspension was then diluted to 4 ml with medium 199, supplemented as previously described (4) for ookinete culture, and incubated for 24 h at 26°C. At the end of the incubation, the cells were washed and extracted with 1% Triton X-100 containing protease inhibitors, immunoprecipitated, and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography conducted as previously described (3).

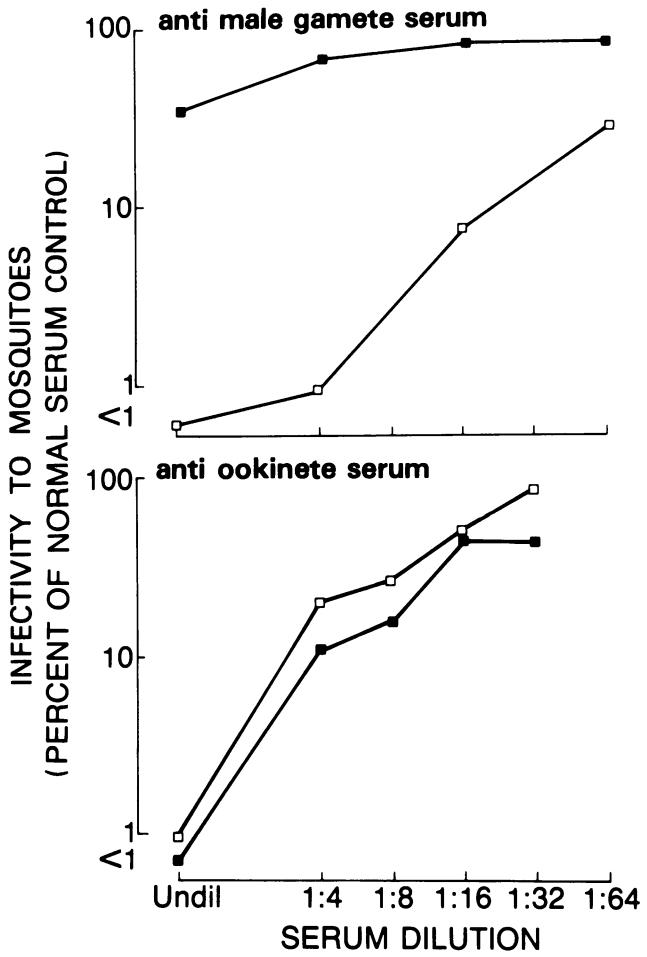

FIG. 1. Effects of stage-specific immune sera raised against male gametes or mature ookinetes on the infectivity of *P. gallinaceum*-parasitized blood to *A. aegypti* mosquitoes. Open symbols represent effects of immune sera administered before gametogenesis and fertilization; solid symbols represent effects of immune sera administered after completion of gametogenesis and fertilization in vitro. Each point is derived from the dissection of 10 experimental mosquitoes and represents the geometric mean number of oocysts per mosquito midgut expressed as a percentage of that found in simultaneous controls.

RESULTS

Preliminary experiments were conducted with heat-inactivated stage-specific sera raised in chickens against either male gametes of *P. gallinaceum* or mature ookinetes of the parasite. These stage-specific antisera were mixed at various dilutions in normal heat-inactivated chicken serum together with chicken blood cells parasitized with *P. gallinaceum* and fed to *A. aegypti* mosquitoes through a membrane feeding apparatus. Both sera efficiently suppressed infectivity of gametocyte-containing blood to the mosquitoes (Fig. 1, open symbols). However, when gametogenesis and fertilization (events which normally occur only after ingestion of the parasites by a mosquito [6]) were stimulated to take place in vitro (7) before exposure of the parasites to antibody, only the anti-ookinete serum continued to suppress infectivity of the fertilized parasites to mosquitoes (Fig. 1, solid symbols).

TABLE 1. Effect of hybridoma-derived antibodies against ookinete surface antigens of *P. gallinaceum* on infectivity of the fertilized parasites to *A. aegypti* mosquitoes

Antibody	Isotype	Infectivity as % of control (mean, range [no. of expts])[a]	Apparent M_r of target antigen	
			Reducing conditions	Nonreducing conditions
IID2	Uncloned	6.4, 3.4–12.0 (10)	28,000	34,000
			26,000	31,000
IID2-B3B3	γ_1	48, 37–64 (6)	28,000	34,000
IID2-E6IV	γ_{2b}	38, 27–54 (3)	28,000	34,000
IID2-C5I	γ_1	8.8, 6.8–11.5 (15)	26,000	31,000

[a] Infectivity of the parasites to *A. aegypti* mosquitoes in the presence of antibodies was measured with *P. gallinaceum*-infected blood prefertilized in vitro. In the control feedings, ascitic fluid from myeloma cell line P3-NS1/1-Ag 4-1 (NS1) (5) was substituted for that containing antiparasitic antibody. Infectivity is the number of oocysts per mosquito midgut for parasites fed in the presence of antibody expressed as a percentage of the number obtained in simultaneous controls. For each antibody, the value given for percent infectivity is the geometric mean of several independent experiments, followed by the 95% confidence limits for the mean derived from the standard errors of the log values of each experiment. The number of replicate experiments for each antibody is given in parentheses.

The properties of the anti-ookinete serum suggested that antigens associated with mature ookinetes are immunogens for antibodies which block infectivity of fertilized parasites to mosquitoes. We therefore derived MAbs directed against surface antigens of ookinetes of *P. gallinaceum*. Hybridoma cell lines secreting antibodies against ookinete surface antigens were identified by indirect immunofluoresence reactions against intact ookinetes. Heat-inactivated ascitic fluids from cell lines giving positive indirect immunofluoresence reactions were tested in membrane feedings for their effect on the infectivity of fertilized parasites to mosquitoes. In the presence of ascitic fluids from one cell line, IID2, infectivity of the fertilized parasites was less than 10% of control levels (Table 1). Cloned lines were derived from IID2 and tested. In the presence of heat-inactivated ascitic fluids (MAbs) from most such lines, e.g., IID2-E6IV and IID2-B3B3 (Table 1), the infectivity of fertilized parasites was only moderately reduced. One line, however, IID2-C5I, suppressed infectivity of fertilized parasites to less than 10% of controls (Table 1) as did subclones derived from IID2-C5I (data not shown). The suppression of infectivity by IID2-C5I was equally effective with parasitized blood in which the antibody was added before initiation of gametogenesis (data not shown).

The MAbs were used to immunoprecipitate from Triton X-100 extracts of zygotes of *P. gallinaceum* biosynthetically labeled with [^{35}S]methionine for 20 h during transformation to mature ookinetes (Fig. 2). A single protein of apparent M_r on nonreducing SDS-PAGE of 31,000 was precipitated by IID2-C5I. All other MAbs derived from IID2, including IID2-E6IV and IID2-B3B3, precipitated a protein of apparent M_r of 34,000 from such extracts. Proteins of apparent M_r 31,000 and 34,000 were also precipitated by the respective MAbs from mature ookinetes of *P. gallinaceum* surface labeled with ^{125}I by the lactoperoxidase method (3) (data not shown). When samples were reduced with β-mercaptoethanol before SDS-PAGE, the apparent M_r of the 31,000 and 34,000 proteins were 26,000 and 28,000, respectively. However, because of the poor resolution of these proteins on SDS-PAGE under reducing conditions, the data are shown here for nonreducing conditions.

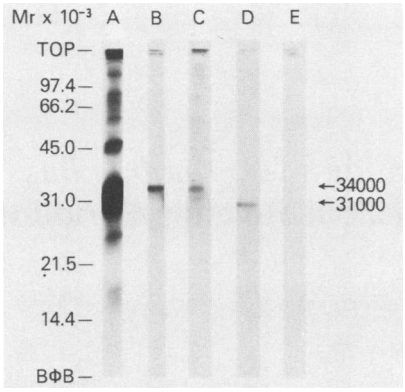

FIG. 2. Immunoprecipitation from Triton X-100 extracts of [^{35}S]methionine biosynthetically labeled ookinetes of *P. gallinaceum*, followed by separation on 12.5% SDS-PAGE under nonreducing conditions; with rabbit anti-ookinete serum (lane A); with MAb IID2-B3B3 (lane B); with MAb IID2-E6IV (lane C); with MAb IID2-C5I (lane D); and with normal rabbit serum (lane E). Under nonreducing conditions, a protein of M_r 31,000 was precipitated by IID2-C5I, and a protein of M_r 34,000 was precipitated by IID2-E6IV or IID2-B3B3. Under reducing conditions, the same proteins ran with apparent M_r of 26,000 and 28,000, respectively (data not shown). Results are given for separation under nonreducing conditions, as resolution of the two ookinete proteins was clearer under these than under reducing conditions.

DISCUSSION

We have identified a protein of M_r 26,000 (31,000 under nonreducing conditions), synthesized by zygotes during transformation to ookinetes and present on the surface of mature ookinetes of *P. gallinaceum*, as the target antigen of a monoclonal antibody which suppresses infectivity of the fertilized parasites to mosquitoes. The mechanism of action and the target antigen of this MAb are clearly distinct from those of antibodies previously identified as preventing fertilization in *P. gallinaceum* (4). Thus, the fertilization-blocking antibodies, which recognize a set of proteins of apparent M_r of ca. 250,000, 60,000, and 55,000 present on male and female gametes (2a, 4), are without effect on the infectivity of the fertilized parasites to mosquitoes. This is in contrast to the MAb which recognizes the protein of apparent M_r of 26,000 synthesized during the transformation of the zygote to an ookinete and which effectively suppresses the infectivity of the fertilized parasites to mosquitoes.

Identification of a second parasite stage in the mosquito midgut, in addition to the gametes of the parasite, as the target of transmission-blocking antibodies has importance for several reasons. These include (i) the increased efficiency of suppression of malaria transmission expected with antibodies affecting sequential stages of parasite development and (ii) the reduced probability that parasites expressing antigenic variants at either the gamete or ookinete stages would escape the combined surveillance of antibodies against both stages.

Further studies are in progress to determine the mechanism of action of antibodies against the zygote protein of M_r 26,000, to characterize its synthesis and expression by the parasites, and to isolate the gene coding for this protein by recombinant DNA technology.

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