SEQUENTIAL EXPRESSION OF ANTIGENS ON SEXUAL STAGES OF *PLASMODIUM FALCIPARUM* ACCESSIBLE TO TRANSMISSION-BLOCKING ANTIBODIES IN THE MOSQUITO

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The induction of transmission-blocking immunity as a potential tool in malaria control was first reported in 1976 by Gwadz (1) and Carter and Chen (2) for the avian malaria parasite *Plasmodium gallinaceum*. Subsequent reports confirmed that the immunogens were present on the surface of both male and female gametes (3, 4). Similar results were reported for a simian malaria parasite, *P. knowlesi* (5), and the murine *P. yoelii* (6). This work was extended to the midgut stages of *P. gallinaceum* by Kaushal and coworkers (7-10). Since the introduction of the in vitro culture for *P. falciparum* (11), good progress has been made in biochemistry and molecular biology of the asexual parasites. The sexual stages, however, demanded special culture conditions. The availability of gametocytes and gametes as well as the establishment of routine in vitro infection of mosquitoes (12) were basic for further studies on this human malarial parasite. In 1983 Rener et al. (13) demonstrated that monoclonal antibodies (mAb)[1] against *P. falciparum* gametes could interfere with the transmission of this parasite to mosquitoes.

We here present results of experiments regarding the nature of antigens on the sexual stages of *P. falciparum*, and their involvement in the blocking of transmission by monoclonal and polyclonal antibodies. These antigens are sequentially expressed in gametocytes, on the surface of gametes, or on ookinetes.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DTT, dithiothreitol; FCS, fetal calf serum; FPLC, fast protein liquid chromatography; IF assay, immunofluorescent assay; mAb, monoclonal antibody; M199, medium 199; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RAM, rabbit anti-mouse.

Materials and Methods

Parasites. Mature gametocytes of *P. falciparum* (isolate NF54, Amsterdam Airport strain) were produced in static cultures (the "tipper" system, 14) as well as in the large scale suspension culture system (15). Parasite suspensions containing gametocytes were harvested 14 d after the initiation of the cultures and centrifuged at 560 g for 5 min. After removal of the supernatant, 10 vol of fetal calf serum (FCS) was added to the pellet and allowed to stand for 30 min at room temperature to allow exflagellation to occur and permit the emergence of the macrogametes. These were then purified using a method modified from Vermeulen et al. (16) (see below).

Transmission Blocking Assays. 14-d-old cultures from the "tipper" system containing fertile gametocytes were used. After removing the spent medium, we washed the residual contents of three culture flasks (4.5 ml) containing ~1 ml of parasitized red cells. This was done by dividing the parasitized cells into two warmed tubes containing 0.5 ml packed red cells, each overlaid by 3 ml RPMI 1640 culture medium without serum. The two tubes were centrifuged at 560 g for 2 min. After discarding the supernatant, the pellets from the two tubes were withdrawn into a prewarmed dosing syringe containing 2 ml of fresh red cells suspended in 3 ml FCS. Before use, the FCS had been stored at $-20°C$ for prolonged periods of time. Very little or no complement activity would have been present in the FCS, after such storage. However, to make sure no active complement was still available, the FCS was inactivated before use in some earlier experiments. Since this treatment had no effect on the outcome, the FCS was used later without inactivation.

The contents of the dosing syringe (~7 ml) was distributed to six membrane feeders. 100 μl of the antigamete antibody in phosphate-buffered saline (PBS) was then introduced and mixed with the mosquito feed material (1–3 mg IgG/ml final concentration). The same volume of PBS was used as a control. *Anopheles stephensi* mosquitoes (Kasur strain) were allowed to feed through the parafilm membrane for 10 min. The fully engorged mosquitoes were held at 26°C for 7 d and their midguts examined for oocysts. In all experiments, at least 10 mosquitoes feeding on the PBS control were included. The degree of transmission block was assessed as the mean percent reduction in the number of oocysts compared with the average in the controls in all experiments with that particular mAb.

Production of mAb. BALB/c mice were immunized with freshly isolated *P. falciparum* macrogametes; 5–10 × 10^6 gametes were injected intraperitoneally together with 10^8 lyophilized *Bordetella pertussis* (National Institute of Health and Environmental Hygiene, Bilthoven, The Netherlands). After 6 mo, 5 × 10^6 macrogametes were given as an intravenous booster 3 d before the fusion. Spleen cells of the immunized mice were fused with myeloma cells of the line P3/X63-Ag 8.653 (17). The fusion protocol was derived from the methods reviewed by Fazekas de St. Groth and Scheidegger (18). A considerable increase in the number of stable hybridomas was achieved by using thymocyte-conditioned HAT (hypoxanthine, aminopterin, thymidine) medium (19). Between days 10 and 14, hybridoma supernates were collected for antibody testing. At the same time three of the four culture trays were frozen according to the procedure of De Ley et al. (20). Hybridomas were selected that satisfied four tests.

The supernates were screened for antibodies specific for *P. falciparum* macrogametes in an immunofluorescent antibody assay (IF assay) with air-dried macrogametes as antigen. The hybridomas with the highest Ig production were selected in a direct enzyme-linked immunosorbent assay (ELISA) using rabbit anti–mouse Ig (see below) as immobilized antibody. The reactivity with surface components of live macrogametes was tested in a suspension IF assay using 10^6 freshly isolated macrogametes and 50 μl hybridoma supernatant. Hybridomas were further selected on the basis of their reactivity with macrogamete proteins on Western blots. Technical details of Western blotting and immune detection are discussed below. Ascites fluid was produced in Pristan-primed BALB/c mice by injecting 1–10 × 10^6 twicely cloned hybridoma cells intraperitoneally.

Usually, mAb were used after 50% ammonium sulfate precipitation of ascites fluid followed by solubilization and extensive dialysis against PBS. For special purposes, however, the IgG fraction was purified directly from ascites fluid using Mono-Q fast protein

liquid chromatography (FPLC) (Pharmacia, Inc., Piscataway, NJ). Separation conditions were as described by Hill et al. (24).

Antisera.   Rabbit anti–mouse IgG (RAM IgG) was produced by injecting New Zealand White rabbits with 2 mg protein A–purified BALB/c IgG in complete Freund's adjuvant given intramuscularly. 5-wk later, 200 μg IgG in incomplete Freund's adjuvant was administered by the same route. The concentration of specific antibodies was checked in a double diffusion assay (25). RAM IgG was used for most purposes as an ammonium sulfate–purified fraction. In specific experiments, however, affinity purification was necessary. Protein A-Sepharose–purified BALB/c IgG was therefore coupled to CNBr-activated Sepharose Cl-4B (Pharmacia, Inc.) according to standard methods (5 mg IgG/ml activated Sepharose). Ammonium sulfate–purified IgG in PBS was allowed to bind in PBS, and the bound material was eluted using 1 M acetic acid.

Rabbit antimacrogamete serum was produced by injecting a New Zealand White rabbit (rabbit 1771) intraperitoneally with 2 doses, 5 wk apart, of 10×10^6 to 20×10^6 purified macrogametes in PBS, and an intravenous third dose, 3 wk later, of 5×10^6 macrogametes.

Purification of Different Parasitic Stages of P. falciparum.   Macrogametes and gametocytes: After 30–45 min incubation in FCS at $25°C$, the parasite suspension contained microgametes, macrogametes/zygotes, immature gametocytes, asexual stages, and uninfected red cells. This suspension was layered on top of a triple layer Nycodenz (Nijegaard, Oslo, Norway) gradient. A 27.6% (wt/vol) Nycodenz stock solution was prepared in 5 mM Tris-HCl (pH 7.5) containing 3 mM KCl and 0.3 mM CaNa$_2$-EDTA. Several Nycodenz concentrations were prepared by dilution of the stock solution with medium 199 (M199), adjusted to pH 7.8 with 0.3 M Tris-HCl of pH 8.0. The gradient consisted of 7-ml layers of 16, 11, and 6% Nycodenz (modified from Vermeulen et al. [16]). The gradient was centrifuged for 30 min at 7,000 g_{max} at $4°C$ in a Sorvall Superspeed 5B centrifuge adapted with a slow-rate accelerator (DuPont Instruments, Newtown, CT) using an Hb-4 swing out rotor. The 6% fraction contained free microgametes, red cell ghosts, pigment, and unidentified debris; the pellet contained asexual parasites and uninfected red cells. Both these fractions were discarded. The macrogametes/zygotes were harvested from the 6–11% interface; the nonactivated gametocytes were found at the 11–16% layer. Cells were washed once with 50 ml of M199 and used for further studies. The macrogamete/zygote suspensions were of high purity, containing only some contaminating red blood cells. In the immature gametocyte fractions, however, some asexual parasites were found.

Schizont-enriched fractions were obtained from 4-d-old suspension cultures by centrifugation on an 18% Nycodenz cushion, under the same centrifugation conditions as above.

Radiolabeling and Solubilization of Macrogamete/Zygote Proteins. 20×10^6 to 50×10^6 purified macrogametes were surface labeled using the lactoperoxidase/glucose oxidase–catalyzed ^{125}I iodination (21, 22). Biosynthetic labeling was performed by incubating 10^7 purified macrogametes/zygotes in 2 ml methionine-free RPMI 1640/10% FCS (pH 7.8) and 200 μCi [^{35}S]methionine (sp act, 1,200 mCi/mmol; Amersham Corp., Arlington Heights, IL) up to 4 h at room temperature. Radiolabeled proteins were solubilized in 25 mM Tris (pH 8.0) plus 0.5% Triton X-100 or Nonidet P-40 (NP-40) (Sigma Chemical Co., St. Louis, MO), pH 8.0 with 1 mM phenylmethylsulfonyl fluoride (PMSF) (stock solution, 100 mM PMSF in 2-propanol) (23) and tested for immunoreactivity as described below.

Immunoprecipitation of Radiolabeled Antigens.   Immune reactions with soluble, radiolabeled antigens were carried out in two ways. (*a*) Indirect immunoprecipitation: 10 μl ammonium sulfate–purified mAb was added to 50–100 μl antigen solution and incubated for 2 h at room temperature. 100 μl protein A–Sepharose was added as a 50% suspension in 25 mM Tris/0.5% Triton X-100/0.5 M NaCl, pH 8.0 and incubated for 30 min at room temperature. Antigen was released from the beads in 50 μl sodium dodecyl sulfate (SDS) sample buffer (0.062 M Tris-HCl, pH 6.8/2.5% SDS), heated for 3 min over boiling water, and analyzed on SDS-polyacrylamide gel electrophoresis (PAGE). For those mAb that did not react with protein A, 50 μg RAM IgG was added and incubated for 30 min at room temperature before the protein A–Sepharose treatment. (*b*) Solid phase immu-

noassay: Ammonium sulfate–purified mAb IgG was coated to polyvinylchloride microtiter plates as described by Tamura et al. (26) at a concentration of 20–100 μg/ml in PBS. The plates were washed with PBS and saturated with 3% bovine serum albumin (BSA) (fraction V; Sigma Chemical Co.) in PBS by incubation for 1 h at 37°C. Radiolabeled antigen was diluted to 0.1% NP-40 with 25 mM Tris/0.5 M NaCl, pH 8.0. Up to 10^5 cpm were added to each well and incubated for 3 h at 37°C or 18 h at 4°C. Plates were washed extensively with PBS/0.05% NP-40. Bound protein was eluted from the plates using SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

SDS-PAGE and Autoradiography. SDS-PAGE was performed using the Laemmli buffer system (27) and gradient gels of 4–20% acrylamide (2.5% crosslinking). Electrophoresis was carried out at 25 mA constant current (V_{max} = 250 V). Gels were either dried for autoradiography or enhanced using 1 M sodium salicylate (28) or Enlightning (New England Nuclear, Dreieich, Federal Republic of Germany) and fluorographed using Kodak XAR-5 film.

Western Blotting and Immunodetection. 50×10^6 to 70×10^6 macrogametes were solubilized in 200–300 μl SDS sample buffer, without the addition of reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol. SDS-PAGE was carried out as described above. Immediately after electrophoretic separation, the slab gel was incubated for 30 min in buffer (0.010 M Tris/0.079 M glycine, pH 8.3). Proteins were transferred electrophoretically to nitrocellulose paper (0.45 μm; Schleicher and Schuell, Inc., Keene, NH) at 10 V/cm (29). For immunodetection (30), nitrocellulose strips (3 mm) were pretreated with 3% BSA in PBS for 1 h at room temperature, and incubated for 3 h with either 50-fold diluted hybridoma supernatants or purified IgG (1000× diluted). After extensive washing with PBS/0.05% Tween-20, the strips were incubated in diluted ^{125}I–protein A or ^{125}I-rabbit anti-mouse–affinity-purified IgG (0.1 μCi/strip). Strips were washed, dried, and autoradiographed against Kodak XAR-5 film using screen amplification. Proteins blotted onto nitrocellulose paper were visualized using India ink according to the procedure described by Hancock and Tsang (31). All protein concentrations were determined according to the Lowry method (32) using BSA and pooled human serum as standard.

Results

Macrogamete Surface Proteins. Surface labeling of macrogametes resulted in a number of ^{125}I-labeled bands after separation on SDS-PAGE. The electrophoretic mobility of some proteins was different in the presence or absence of reducing agents like DTT. In Fig. 1a, a typical labeling pattern is shown. Usually, four major polypeptides could be detected. Lane 1 contains these total labeled polypeptides in nonreduced state (M_r 230, 48/45, 43, and 10 K). Under reducing conditions the above proteins demonstrated an M_r of 250, 58, 47, and 10 K, respectively (Fig. 1a, lane 2). The doublet at M_r 48/45 K did not separate under reducing conditions but migrated as one band at 58 K (see also lanes 5–8). Minor bands were seen at M_r 33, 25, and 16 K (M_r under nonreducing conditions will be used in referring to the various proteins). mAb 32F1, 32F3, and 32F5 reacted with the M_r 48/45 K doublet (Fig. 1a, lanes 3–8). 32F1 coprecipitated the M_r 230 K protein (Fig. 1a, lanes 3 and 4). Fig. 1b shows the ^{125}I-labeled target proteins in the reduced state. mAb 28F1 reacted only with the M_r 230 K protein (250 K, Fig. 1b, lane 1). mAb IIC5-B10 (gift from Dr. R. Carter) also precipitated the M_r 48/45 K proteins (58 K, Fig. 1b, lane 2). The reaction of the polyvalent rabbit serum (1771) is shown in Fig. 1b, lane 3.

Immunodetection on Western Blots. Immunoblotting experiments using polyclonal as well as monoclonal antibodies showed a somewhat different result (Fig. 2). Polyclonal rabbit serum (Fig. 2, lane 1) did not bind to the M_r 230 K protein.
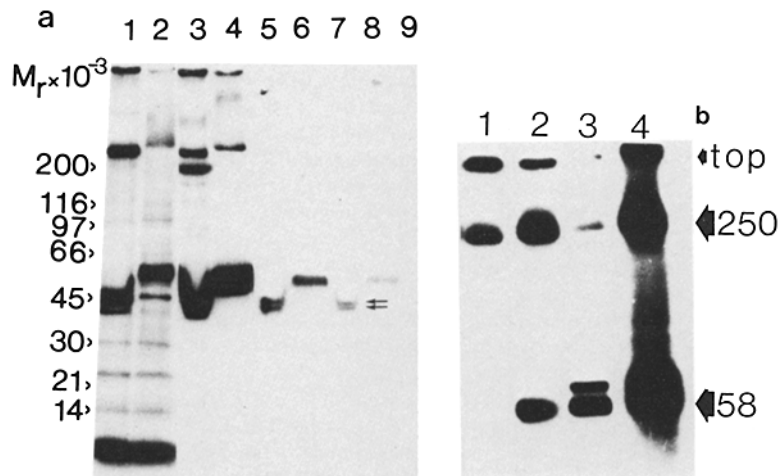
FIGURE 1. (a) SDS-PAGE of ^{125}I surface-labeled proteins of *P. falciparum* macrogametes precipitated by different mAb. (Lane *1*) Total labeled proteins under nonreducing conditions (*NR*). (*2*) Same as lane *1*, but reduced. (*3*) Immunoprecipitated by mAb 32F1 (*NR*). (*4*) Same as lane *3*, but reduced. (*5*) Immunoprecipitated by 32F3 (*NR*). (*6*) Same as lane *5*, but reduced. (*7*) Immunoprecipitated by 32F5 (*NR*); arrows indicate the M_r 48/45 K doublet. (*8*) Same as lane *7*, but reduced. (*9*) PBS control. (*b*) SDS-PAGE of ^{125}I surface-labeled proteins of *P. falciparum* macrogametes precipitated by mAb and polyclonal rabbit serum. (Lane *1*) Immunoprecipitated by mAb 28F1. (*2*) Immunoprecipitated by mAb IIC5-B10 (gift from Dr. R. Carter; see also Ref. 9). (*3*) Immunoprecipitated by rabbit serum 1771. (*4*) Total labeled proteins. Arrows indicate the apparent molecular weight of the relevant proteins (under reducing conditions).

On the other hand, the same serum reacted prominently with an M_r 25 K protein that was not precipitated after surface iodination. mAb that reacted with the 25 K protein were selected by immunoblotting (Fig. 2, lanes 3, 6, 7, and 10). Similarly, it was possible to select mAb reacting with an M_r 10 K protein that also recognized a 45 K protein (Fig. 2, lane 4, 8, and 9).

Transmission Blocking Assays. Seven transmission blocking and eight nonblocking antimacrogamete antibodies were identified (Table I). The molecular weights of the proteins of macrogamete/zygotes recognized by the mAb and polyclonal rabbit serum are also included as a reference. Table II gives information on the potency of the different blocking antibodies. mAb 32F3, the most powerful inhibitor of oocyst development, blocked transmission totally in all 11 experiments, involving 173 mosquitoes. Among the controls, the mean oocyst number per dissected mosquito was 24. The weakest among the blockers was 32F61, which reduced oocyst numbers by 74% in 53 mosquitoes in four experiments. The mean oocyst count in control mosquitoes was 16. The remaining five blocking antibodies inhibited oocyst formation >95% in experiments in which the lowest mean oocyst number in control mosquitoes was 14.

To discover whether the blocking action of one mAb could be potentiated by mixing with another, mAb were used in dilutions that on their own could not totally inhibit transmission. In two experiments, mAb 32F1 (nonblocker, anti-230 and -48/45 K M_r proteins) was combined with 32F3 (blocker, anti-48/45 K M_r proteins). Table III shows the results of the blocking activity of the combi-
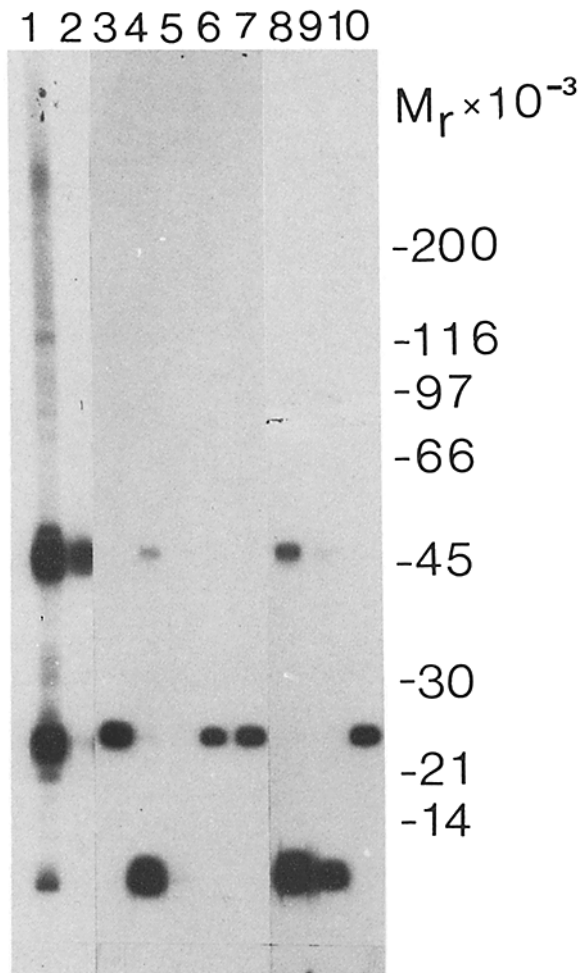
FIGURE 2. Western blotting and immunodetection of *P. falciparum* macrogamete proteins (nonreduced). The following antibody preparations were used: (Lane *1*) Rabbit serum 1771. (*2*) mAb 32F3. (*3*) 32F61. (*4*) 32F62. (*5*) PBS control. (*6*) 32F71. (*7*) 32F72. (*8*) 32F63. (*9*) 32F84. (*10*) 32F81.

nations as well as the individual, diluted FPLC-purified mAb. The results of transmission blockade using ammonium sulfate–purified mAb were similar (data not shown). 32F3, which blocks totally at the normal concentration of 1–3 mg protein/ml, was unable to block at both 20-fold and 40-fold dilutions. In the first experiment a 1:20 dilution of 32F3 combined with 32F1 (nonblocker) increased the inhibition from 63 to 97% and, in the second experiment, from 74 to 85%.

32F3 (dilution, 1:40) and 32F81 (1:10), when used individually, reduced oocyst formation by 36 and 89%, respectively. However, in the same experiment but in which the two mAb were mixed, the inhibition in the number of oocysts was increased to 98%. In the second experiment the combination resulted in 94% oocyst reduction. Combinations of nonblocking mAb recognizing proteins of the

TABLE I

Action of a Selected Number of Monoclonal and Polyclonal Antibodies Directed Against P. falciparum Macrogametes/Zygotes

		Molecular weight of target proteins			
		230 K	48/45 K	45/10 K	25 K
Transmission-blocking antibodies	7F11				+
	29F432	+	+		
	32F3		+		
	32F5		+		
	32F61				+
	32F81				+
	1771*	+	+	+	+
Nonblocking antibodies	7F3	+	+		
	28F1	+			
	32F1	+	+		
	32F62			+	
	32F63			+	
	32F71				+
	32F72				+
	32F84			+	

* Rabbit polyclonal antibody.

TABLE II

Degree of Transmission Block by Monoclonal and Polyclonal Antibodies Directed Against P. falciparum Macrogametes/Zygotes

Antibody	Mean percent reduction in oocysts compared with control	Total No. of test mosquitoes dissected	Mean oocysts in control mosquitoes
7F11	97 (3)	79	17
29F432	97 (2)	25	21
32F3	100 (11)	173	24
32F5	95 (5)	103	14
32F61	74 (4)	53	16
32F81	95 (6)	74	18
1771*	99 (4)	76	19

The number in parentheses indicates the number of experiments carried out with each antibody.
* Rabbit polyclonal antibody.

same molecular weight or different molecular weights had no effect on the transmission when compared with controls (data not shown).

Biosynthesis of the M_r 25 K Protein. To investigate whether and, if so, when the M_r 25 K protein was de novo synthesized, freshly purified macrogametes were incubated in methionine-free RPMI 1640 supplemented with [^{35}S]methionine. The proteins labeled during 3 h of incubation at 25°C are shown in Fig. 3, which shows a dominant band at M_r ~25 K. This protein could be solubilized almost completely using 25 mM Tris/0.5% Triton X-100, pH 8.0 (Fig. 4). Fig. 4, lane 2 contains the total protein labeled in 3 h; the Triton-solubilized fraction is shown in lane 3 and the residual proteins in the pellet after Triton extraction

TABLE III

Transmission Blocking Activity of Dilutions of FPLC-purified mAb and their Combinations

		32F1	32F3	32F1 + 32F3		32F3	32F81	32F3 + 32F81	
	mAb:	32F1	32F3	32F1 + 32F3		32F3	32F81	32F3 + 32F81	
	Transmission blocking (+/−)	−	+	−	+	+	+	+	+
	Dilution:	1:10	1:20	1:10	1:20	1:40	1:10	1:40	1:10
Infectivity rate*	Exp. 1	18/19	16/23	3/15		22/24	5/9	3/14	
	Exp. 2	6/7	4/8	5/7		9/10	5/11	8/9	
Mean oocyst per mosquito	Exp. 1	11.4	4.2	0.4		7.3	1.3	0.2	
	Exp. 2	46	11.8	6.9		15.9	8.5	2.7	
Mean percent reduction in oocysts compared with control	Exp. 1	0	63	97		36	89	98	
	Exp. 2	0	74	85		65	82	94	

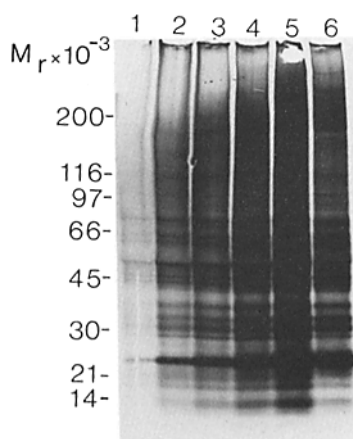* Infectivity rate: number of positive mosquitoes per number dissected.

FIGURE 3. Nonreduced SDS-PAGE of [^{35}S]labeled proteins synthesized by purified *P. falciparum* macrogametes/zygotes up to 3 h after isolation. (Lane *1*) After 15 min of labeling. (*2*) 30 min. (*3*) 60 min. (*4*) 90 min. (*5*) 120 min. (*6*) 180 min.

in lane 1. Fig. 4, lane 4 shows that the prominent band at M_r 25 K is indeed precipitated by 32F61.

Similar results were obtained with the transmission blocking mAb 32F81 and 7F11 as well as the nonblockers 32F71 and 32F72 (see also Table I). Gametocytes synthesized the M_r 25 K protein at a very low but detectable level. Although [^{35}S]methionine labeling appeared too weak to immunoprecipitate detectable quantities, the presence of the M_r 25 K protein in gametocytes was demonstrated on Western blots. To avoid any contamination with macrogamete proteins, gametocytes were harvested in the presence of 1 mg/ml emetine/HCl, which blocks protein synthesis irreversibly. Equal numbers of both gametocytes and macrogametes were applied to SDS-PAGE. A similar number of asexual parasites were also included for comparison. The proteins were separated and blotted onto nitrocellulose paper. The blots were screened for reaction with 32F61 (Fig. 5*A*), with a combination of 32F61 and 32F3 (Fig. 5*B*) and of 32F71 and 32F63 (Fig. 5*C*). Whereas the intensity of the reaction to the M_r 45 K protein was equal
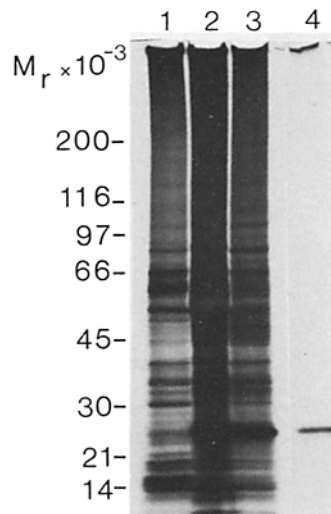
FIGURE 4.   Nonreduced SDS-PAGE of ^{35}S-labeled macrogamete/zygote proteins solubilized in Triton X-100 and precipitated by mAb 32F61. (Lane *1*) Nonsolubilized proteins after Triton extraction. (*2*) Total labeled proteins before extraction. (*3*) Triton-soluble fraction. (*4*) M_r 25 K protein precipitated by mAb 32F61.
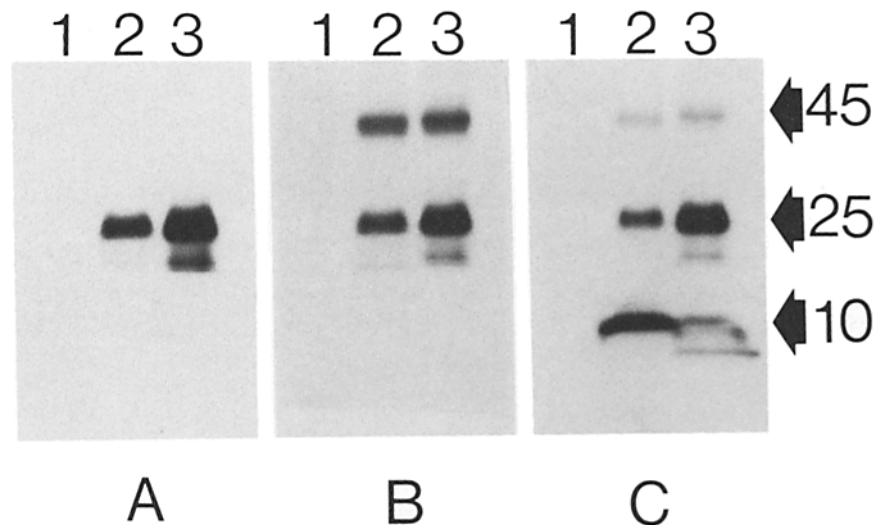

FIGURE 5.   Comparison of the presence of M_r 48/45 and 25 K proteins in *P. falciparum* using Western blots of extracts from (*1*) asexual parasites, (*2*) gametocytes, (*3*) macrogametes. Blots were screened using the following mAb: (*A*) 32F61. (*B*) 32F61 plus 32F3. (*C*) 32F71 plus 32F63.

in both the sexual stages, the 25 K protein was more prominent in macrogametes than in gametocytes. Asexual stage proteins did not react with any of the antibodies used. The M_r 25 K protein is present in gametocytes, but is predominantly synthesized in macrogametes/zygotes and is a target of transmission blockade of extracellularly present mAb. This suggested that, in the midgut, the M_r 25 K protein is expressed later on the surface of the stages, during the development from zygote to ookinete. This was tested by surface immunofluo-

rescence of midgut stages using different mAb. These developmental stages were obtained from mosquito midguts 24 h after the infective feed. They were easily identified, since gametocytes, asexual blood forms, and noninfected red blood cells had already been digested at that time. Fig. 6 shows the fluorescence of the three undigested forms using mAb 32F71. Fig. 6a shows the fluorescence of an undeveloped macrogamete/zygote; Fig. 6b shows the intermediate stage of the developing ookinete (a so-called retort cell); in Fig. 6c the ookinete is fully mature. The mAb reacting against the surface components of these stages precipitate only the M_r 25 K protein.

Transmission Blocking Action of mAb.   To investigate the effect of mAb on the development of ookinetes, mosquitoes were allowed to feed on mature gametocyte cultures to which blocking mAb 32F3 or 32F81 were added. Some of the mosquitoes were dissected 24 h later, their midguts homogenized, and ookinetes counted in a hemocytometer. The remainder was held at 26°C for 1 wk and oocyst numbers determined. The data in Table IV show that the mode of action of the two mAb is different. 32F3 drastically blocks ookinete formation. 32F81,
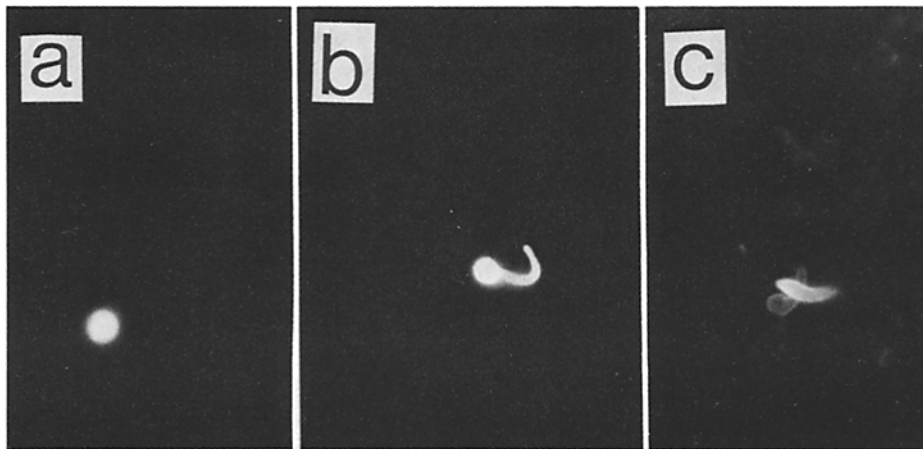

FIGURE 6.   Surface immunofluorescence of different stages isolated from mosquito midguts 24 h after the feeding with fertile *P. falciparum* gametocytes. All stages were incubated "live" with mAb 32F71 (anti-M_r 25 K protein) and FITC-labeled goat anti–mouse IgG. (A) Undeveloped macrogametes/zygotes. (B) Intermediate "retort cell". (C) Mature ookinete. None of these stages showed any surface fluorescence with mAb 32F3 or 32F63.

TABLE IV

Influence of Transmission Blocking mAb on the Development of Ookinetes

		PBS	32F3	32F81
Exp. 1	Retort cells plus ookinetes*	1,560 ± 480	0.0	800 ± 380
	Oocysts‡	7.0 ± 5.7	0.0	0.0
Exp. 2	Retort cells	1,180 ± 500	20 ± 60	520 ± 350
	Ookinetes	2,060 ± 1,000	40 ± 80	1,260 ± 420
	Oocysts	22 ± 32	0.0	0.9 ± 2

All data are based on 10 dissected mosquitoes per group, except for the ookinete/retort cell counts in Exp. 1 ($n = 5$).
* Mean number of cells per mosquito ± SE.
‡ Mean number of oocysts per dissected mosquito ± SE.

however, interferes at a later stage before oocyst formation. In experiment 1 retort cells and ookinetes were not separately counted. The apparent reduction in ookinete and retort cell numbers by 32F81 was not significant by Wilcoxon's test.

Discussion

Fertile gametocytes ingested by a mosquito vector undergo several developmental changes in the insect's midgut before oocysts are formed. The extracellular gametes, zygotes, and ookinetes could become the target of antibodies taken up by the mosquito together with the sexual stages. Antigens on the sexual stages of *P. falciparum* were identified using mAb antibodies. The strength of transmission block depends on two factors, the concentration of the specific antibodies and the quality of the gametocytes involved. Although the concentration of mAb was relatively high in the transmission blocking assays, the number of infectious gametocytes ($\sim$20,000/mm^3) was much higher than in normal field conditions (33). The effect of combinations of antigamete mAb was first demonstrated by Rener et al. (34) with avian malaria. Later they showed a similar phenomenon in *P. falciparum* (13). In our system, although complete transmission inhibition could be achieved by a single mAb, combinations of mAb could also potentiate transmission block.

Target Proteins. The SDS-PAGE pattern of surface-labeled proteins of *P. falciparum* NF54 macrogametes was similar to that described by Rener et al. (13) for a Brazilian isolate. Some minor differences were apparent, such as the intense labeling of 8 to 10 bands in Rener's system as compared with four (M_r 230, 48, 45, and 10 K) in ours. This is probably due to technical differences in the isolation procedure and labeling conditions. Another factor that could influence the labeling result is the dynamics of protein expression on the surface of the macrogamete/zygote. This is a relatively short-lived intermediate form, the surface of which changes during the transition from the gametocyte to ookinete. We confirm the finding of Rener et al. (13) that some labeled proteins showed a difference in migration in SDS-PAGE under reducing and nonreducing conditions.

mAb were selected on the basis of their binding to proteins of M_r 230, 48/45, 45/10, and 25 K. These proteins were shown to be present already in the gametocyte stage, but were expressed on the surface of the parasite only later in development.

M_r 230 K Protein. This protein could be labeled on the surface of the freshly emerged macrogamete. It was precipitated by the polyclonal rabbit serum (1771) as well as by some mAb, such as IIC5-B10 (13), 32F1, 29F432, and 7F3, but always together with the smaller proteins of M_r 48/45 K. Only one mAb (28F1) was monospecific for the M_r 230 K protein. These results suggest that these three proteins could possess a common antigenic site and that they are interrelated. This relationship, however, is still very questionable. It appears that the quantity of the M_r 230 K protein precipitated relative to the quantity of lower M_r proteins differs from one mAb to another. IIC5-B10, raised against the Brazilian isolate, bound equal amounts of high and low M_r protein (in our NF54 system). On the other hand, 32F1, 29F432, and 7F3 bound significantly more

of the lower M_r proteins. This suggests that the M_r 230 K protein probably coprecipitates in a nonspecific way. Another observation favoring this explanation is that, in the immunoblotting experiments, a positive reaction to the M_r 230 K protein could never be detected using any of the above-mentioned mAb or the polyclonal serum. That this lack of positive immunoblotting reaction is not due to a low transfer efficiency of the M_r 230 K protein is shown by the fact that intense bands up to 400 K were demonstrated by staining the nitrocellulose blots with India ink. The importance of the M_r 230 K protein in the transmission blocking action of these mAb is also not clear. mAb 28F1 (monospecific for the M_r 230 K protein) exhibited no transmission blocking activity. The M_r 230 K protein was not detectable by IF assay on the surface of developing ookinetes.

Using synchronized gametocyte cultures we have studied the biosynthesis of the M_r 230, 48, 45, 25, and 10 K proteins. Pulse chase experiments clearly indicated that no precursor-product relationship exists between any of the above proteins (Vermeulen, Van Deursen, Ponnudurai, and Brakenhoff, manuscript in preparation). These results corroborate the studies by Kumar and Carter (35).

M_r 48/45 K Doublet. The significance of these two proteins in the development of the zygote to the ookinete stage has become very clear. mAb such as 32F3 and 32F5, binding to an epitope of these proteins, were able to block oocyst formation effectively. The proteins are already present in the gametocyte but are later expressed on the surface of the newly emerged macrogamete. Similar to the M_r 230 K protein, this set of proteins could no longer be detected on the intermediate stage (retort cell) between zygote and ookinete. Because of the above and due to the fact that 32F3 completely blocked the formation of retort cells/ookinetes, it appears that these proteins play a significant role in the process of fertilization by the microgamete. Using surface iodination, Carter and Kaushal (10) could not detect any protein of M_r >50 K on transforming zygotes of *P. gallinaceum*. These antigens were found unaltered in the extracellular fluid. It is very likely that a similar situation exists in *P. falciparum* and that the M_r 48/45 K proteins are shed during ookinete development.

The biologically significant epitopes of this set of proteins were not sensitive to the action of SDS, as was demonstrated by immune reaction on Western blots. These epitopes, however, were sensitive to reduction by DTT. The reduced proteins not only showed a difference in molecular weight, but the reactivity of these blotted (DTT-treated) proteins was abolished (data not shown). Blocking mAb as well as nonblockers did not bind to these reduced proteins on blots, suggesting that these epitopes have a conformational nature. Transmission blocking activity was increased by the combination of nonblocking mAb 32F1 with the blocker 32F3. Since both mAb recognize the same proteins, the potentiation of the blocking activity could have occurred only if the epitopes are different. Crossreactivity could not be detected between epitopes of a blocker (32F3) and a nonblocker (32F1) in experiments using an immunoradiometric assay modified from Zavala et al. (36). These experiments also demonstrated that the target epitopes of the blocking mAb were not repeated (37).

M_r 45/10 K Set. This set of proteins could also be detected in the gametocyte stage. mAb against these two proteins (32F62, 32F63, and 32F84), selected using Western blots, also showed a positive immunofluorescence on live gametes.

However, none of these mAb could precipitate any surface-iodinated bands or block oocyst formation in the mosquito. The significance of these proteins is therefore not yet clear. It was remarkable that a considerable number of the hybridoma lines produced antibodies directed against this set of proteins, suggesting that they are very immunogenic. The M_r 10 K protein is predominantly present with respect to the M_r 45 K one. From preliminary experiments using two-dimensional PAGE it appeared that the M_r 45 K protein is identical to that reacting with mAb 32F3. Reduction of this 45 K protein by DTT resulted also in a shift in M_r in SDS-PAGE to 58 K. The epitope present on both the M_r 45 and 10 K proteins was, however, not sensitive to reduction (results not shown).

M_r *25 K Protein.*   This protein is already present in mature gametocytes. That this was not due to contamination with proteins synthesized by gametes was confirmed by the use of emetine treatment. Emetine blocks protein synthesis irreversibly. The suspended animation (SA) medium of Carter et al. (3) could not be used for this purpose since it was impossible to prevent the activation of gametocytes in the presence of this buffer. Preliminary experiments further indicate that the M_r 25 K protein is already being synthesized in small quantities in early stages of gametocyte development.

mAb against this protein were selected on Western blots of macrogamete proteins. Some mAb directed against this protein were able to block transmission (7F11, 32F61, and 32F81). Nonblocking mAb probably bind to another determinant on the molecule. This was recently demonstrated using the two-site immunoradiometric assay (37). From these studies it also appeared that the transmission blocking epitopes on the M_r 25 K protein are monovalently expressed (37).

Thus, besides the 48/45 K set, the 25 K protein also plays an important role in the parasite development. Using surface immunofluorescence with mAb on live cells, we demonstrated that this protein is hardly detectable on the surface of newly emerged macrogametes, but that it becomes progressively expressed on the surface of the macrogamete/zygote over a period of 2 h. This also implies that this protein is probably not involved in the fertilization process, but has a function after the formation of the ookinete. This was confirmed by the fact that the presence of mAb 32F81 in the mosquito bloodmeal did not significantly influence the number of retort cells/ookinetes formed. Although the antibodies were bound to the surface of the transforming zygote from ~2 h after the feed, they obviously did not hamper the development of the ookinete significantly. It is suggested that the M_r 25 K protein acts as a ligand for the receptor on the mosquito midgut cell wall and is therefore important for the penetration of the ookinete.

Although detectable by IF assay on the surface of macrogametes/zygotes, the M_r 25 K protein was not iodinated, probably due to a lack of tyrosine/histidine residues exposed. Using surface iodination of *P. gallinaceum* transforming zygotes, Carter and Kaushal (10) demonstrated two proteins of M_r 26 and 28 K under reducing conditions. Nonreduced, these proteins had M_r of 31 and 34 K, respectively. It is difficult to produce *P. falciparum* ookinetes in vitro (Sinden and Verhave, personal communication). We have therefore not attempted surface iodination of this stage.

The similarity between *P. falciparum* and *P. gallinaceum* is striking with regard to the expression of surface proteins during gametogenesis and subsequent development of ookinetes. Not only are the molecular weights of the proteins in the two parasite species very similar, but apparently also their function and fate. Perhaps this resemblance in protein expression is not coincidental but due to the close relationship between avian plasmodia and *P. falciparum*, as has been suggested both on morphological criteria and genomic organization (38, 39). The polyclonal and monoclonal antibodies used in this study are now being used for screening *P. falciparum* genomic/cDNA libraries. Based on the results described in this paper it is our belief that the M_r 48/45 and 25 K proteins are good candidates for a future transmission blocking vaccine. Such a vaccine containing a combination of epitopes from both proteins will ensure the blocking of transmission even when the transmission potential in the population is high.

Summary

Plasmodium falciparum gametocytes contain specific antigens, some of which (M_r 230,000, 48,000, 45,000) are expressed on the surface of the newly emerged macrogamete. A different antigen (M_r 25,000) surrounds the surface of the ookinete and, although present to some extent in the developing gametocyte, is synthesized in high quantities by the macrogamete/zygote and expressed progressively on the transforming zygote surface. These antigens are targets of transmission blocking antibodies that are effective at two distinct points after gametogenesis: fertilization of the macrogamete and ookinete to oocyst development.

The antigens involved in the fertilization blockade are the M_r 48 and 45 proteins, which are expressed on the macrogamete surface. The M_r 230 K coprecipitating protein probably plays no part in transmission block. mAb directed against the M_r 25 K ookinete surface protein blocked transmission without inhibiting ookinete formation, indicating that this protein has an important role in the transformation of ookinete into oocyst. A combination of mAb recognizing different epitopes on the same protein molecule acted synergistically in inhibiting oocyst formation. Using a mixture of two blocking mAb reacting against the M_r 48/45 and 25 K proteins, respectively, an additive blocking effect could be demonstrated.

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