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Two Antigens on Zygotes and Ookinetes of *Plasmodium yoelii* and *Plasmodium berghei* That Are Distinct Targets of Transmission-Blocking Immunity

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We have developed transmission-blocking monoclonal antibodies (MAbs) against *Plasmodium yoelii* 21-kDa (Pys21) and 28-kDa (Pys25) ookinete surface proteins. These MAbs block infectivity of *P. yoelii* to *Anopheles stephensi*. One MAb, 14, cross-reacted by Western blotting with a 28-kDa surface protein (Pbs25) of *P. berghei* ookinetes and blocked oocyst development, as assayed by direct mosquito feeds on passively immunized *P. berghei*-infected mice. In total, we have identified two ookinete surface proteins in *P. yoelii*, one of which is also present in *P. berghei*. The transmission-blocking activity of the anti-Pys25 MAb 4 was complete and more potent than that of the anti-Pys21 MAb 2. Moreover, Fab fragments of MAb 4 had transmission-blocking activity in mice. In comparison, Fab fragments of MAb 2 did not have detectable transmission-blocking effect, although $F(ab')_2$ did. Furthermore, MAb 2 and MAb 4 appeared to block the in vitro formation and development of zygotes as well.

Malaria control strategies include the prevention of transmission of the parasite from the mammalian host to the *Anopheles* mosquito vector. The targets of this blockade are the *Plasmodium* sexual stages. Several sexual-stage proteins can elicit antibodies capable of preventing parasite transmission either by interfering with fertilization or by preventing development of the postfertilization stages (1). In the postfertilization stages, target antigens of 25 and 28 kDa have been reported. The 25-kDa transmission-blocking target protein of *Plasmodium falciparum*, Pfs25, represents a potential candidate for a transmission-blocking vaccine (9). Pgs25 (5, 10), an analog of Pfs25, is present in the avian malaria parasite, *Plasmodium gallinaceum*, which has phylogenetic proximity to *P. falciparum* (23). The 28-kDa protein, Pgs28, is present in *P. gallinaceum* (3, 5), and an analog, Pbs21 (14), is present in the rodent malaria parasite, *Plasmodium berghei*. Recently, Pfs28, the analogous antigen in *P. falciparum*, was identified (2). All of these antigens have epidermal growth factor-like, cysteine-rich, lipid-anchored structures.

Studies of the immune responses to these ookinete surface proteins and a search for and identification of B- and T-cell epitopes in these target antigens are being carried out. To elucidate the immune effector mechanism(s) of transmission-blocking immunity in vivo, the rodent malaria parasite has some advantages over the avian or human malaria parasite, particularly because of the accumulated knowledge of murine immunity, access to a variety of well-characterized genetic variants of mouse immune response genes, and reasons of safety. However, only one cysteine-rich ookinete surface protein, Pbs21 from *P. berghei*, has been previously characterized for rodent malaria parasites (14). Here we present data on the identification of analogous proteins of *Plasmodium yoelii* and *P. berghei* by using monoclonal antibodies (MAbs) raised against *P. yoelii* ookinetes which have been shown to block infectivity of the parasites to mosquitoes.

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MATERIALS AND METHODS

Parasites. *P. yoelii* 17X (lethal) and *P. berghei* ANKA were maintained by mosquito transmission in *Anopheles stephensi* interspersed by a maximum of two serial passages in Crj:CD-1 (ICR) mice purchased from Charles River Japan, Inc. (Yokohama, Japan). In some experiments, we used C5-deficient DBA/2NCrj (DBA/2) mice (13) purchased from Charles River Japan. DBA/2 mice were also useful for mosquito live feeds, because the number of oocysts formed in mosquito midgut was much higher than for the other mouse strains (21). The animal experiments in this study were carried out in compliance with the Guide for Animal Experimentation at Ehime University School of Medicine.

Ookinete culture. Female ICR mice, 8 weeks of age, were infected with *P. yoelii* (10^6 parasitized cells per mouse) by intraperitoneal injection and bled 4 days later into heparinized syringes (100 μg/ml of blood) by cardiac puncture under ether anesthesia. After passage through a sterile cellulose CF11 (Whatman, Maidstone, England) column to remove leukocytes, the infected blood was diluted in phosphate-buffered saline (PBS; pH 7.4) to a hematocrit of 10% and layered onto a 48% (vol/vol) Nycodenz (Nycomed Pharma, Oslo, Norway)-PBS cushion. After centrifugation ($350 \times g$ for 25 min at room temperature), the gametocyte-rich layers were collected at the interface and washed three times in PBS. The gametocytes were then diluted 1 to 10 with RPMI 1640 (Gibco BRL, Gaithersburg, Md.) medium supplemented with 50 μg of hypoxanthine per ml, 25 mM HEPES, 20% heat-inactivated fetal calf serum (Gibco BRL), 24 mM $NaHCO_3$, 5 U of penicillin per ml, and 5 μg of streptomycin per ml (pH 8.4) and cultured for 24 h at 24°C in air. In some experiments, the purified gametocytes were cultured with purified MAbs or Fab fragments. After being washed with PBS, the cultured ookinetes were suspended in the same volume of PBS as the ookinete medium, and 4-μl aliquots were spotted onto Multitest slides (Flow Laboratories, McLean, Va.) for indirect immunofluorescence assay (IFA). The slides were stained with MAb 4, and then the numbers of zygotes and ookinetes were counted by using IFA. The differences in the number of the parasites between the control and MAb groups were compared by the Mann-Whitney *U* test. The cultured ookinete pellets were stored for Western blotting and immunization. To prepare ookinetes of *P. berghei*, ICR mice were pretreated with phenyl-hydrazine (Wako, Osaka, Japan) at 6 mg/ml in PBS in a single intraperitoneal injection of 200 μl 3 days before infection. Gametocytes were subsequently purified and cultured in the same manner as *P. yoelii* except that the temperature of cultivation was 19°C.

Antibodies. BALB/c mice (Charles River Japan) used to produce MAbs were immunized by intraperitoneal injection of an emulsion of Freund's complete adjuvant (Wako) and 100 μg of parasite protein containing a mixture of 24-h-old gametes/zygotes, retorts (the intermediate stage between zygotes and mature

ookinetes), and mature ookinetes in 100 μl of PBS. Three weeks later, the mice were boosted with the same dose emulsified in Freund's incomplete adjuvant (Wako); 3 weeks later, a mouse selected for fusion was boosted intravenously with the same amount of parasite protein in PBS. MAbs were prepared essentially as described by Harlow and Lane (6) and screened by IFA of acetone-fixed parasites. After an initial screening, several hybridoma cell lines were cloned by two rounds of limiting dilution. Selected subclones were expanded for freezing and for ascites production in pristane-primed mice. The subclass of each hybridoma subclone supernatant was determined by a mouse MAb isotyping kit (Amersham, Buckinghamshire, England). Immunoglobulins were purified from ascites fluid by using an Ampure PA kit (Amersham).

Preparation of Fab fragments of MAbs. MAbs were digested with papain by the method of Mage (12). Briefly, 2 to 4 mg of the purified MAb dissolved in 0.2 ml of 0.2 M PBS containing 5 mM EDTA and 25 mM mercaptoethanol was digested by adding papain (25 μg; Sigma, St. Louis, Mo.) followed by incubation for 1 h at 37°C. After the incubation, the reaction was stopped by adding iodoacetamide to a final concentration of 30 mM and incubating the mixture for an additional 15 min at 37°C. The mixture was desalted on a PD-10 column (Pharmacia Biotech, Uppsala, Sweden) into 25 mM phosphate buffer (pH 6.0) containing 0.02% NaN_3 and then applied to a Mono Q column, using a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech). The unbound fractions from the Mono Q column were concentrated, and then the buffer was changed to PBS (pH 7.4) by using a PD-10 column. The final purity of the Fab fragments of MAbs was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Preparation of $F(ab')_2$ fragments of MAbs. One milligram of the purified MAb dissolved in 0.2 ml of 0.1 M citrate-Tris buffer (pH 3.6) containing 0.02% NaN_3 was digested by adding pepsin (20 μg; Sigma) followed by incubation for 24 h at 37°C. After the incubation, the reaction was terminated by raising the pH to >7 with 1 M Tris (pH 10.5). The digestion mixture was applied to a Superdex 200 column by using an FPLC system (Pharmacia Biotech), and then the $F(ab')_2$ fraction eluted at a molecular mass of 100 kDa was dialyzed against 20 mM PBS (pH 7.4). The final purity of the $F(ab')_2$ fragments of MAbs was confirmed by SDS-PAGE.

Indirect immunofluorescence microscopy. Air-dried parasites on Multitest slides prepared as described above were fixed with ice-cold acetone and then blocked with PBS containing 5% nonfat dry milk for 30 min at 37°C. The slides were incubated with diluted MAb for 60 min at 37°C and rinsed with PBS. After incubation with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) plus IgM (Tago, Camarillo, Calif.) serum for 30 min at 37°C, the slides were rinsed with PBS and mounted under a coverglass in bicarbonate-buffered glycerin and observed with a fluorescence microscope (Olympus, Tokyo, Japan).

Immunoelectron microscopy. Cultured ookinetes were fixed in 1% paraformaldehyde–0.1% glutaraldehyde in 0.1 M phosphate buffer and embedded in LR white resin (Polysciences, Inc., Warrington, Pa.). Sections were blocked for 30 min in 0.1 M PBS containing 5% nonfat dry milk and 0.01% Tween 20 (PBS-milk-Tween), incubated overnight at 4°C in PBS-milk-Tween containing mouse anti-ookinete MAbs or control MAb, and then incubated for 1 h in PBS-milk-Tween containing goat anti-mouse IgG conjugated to gold particles (Jansen, Piscataway, N.J.). Sections were stained with 2% uranyl acetate in 50% methanol and examined with a Hitachi H-800 electron microscope.

Western blotting. Cell pellets of ookinetes were extracted with SDS-PAGE loading buffer, the extracts were size fractionated under nonreducing or reducing conditions on SDS–5 to 20% gradient polyacrylamide gels (11), and proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) as described previously (19). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; pH 7.4). Strips were incubated for 1 h at room temperature with MAb diluted 1/200 in 0.05% Tween 20 in TBS (TBST). After extensive washing with TBST, the strips were incubated with biotin-conjugated rabbit anti-mouse IgG plus IgM plus IgA (Nichirei, Tokyo, Japan) diluted 1/1,000 in TBST. After extensive washing with TBST, the strips were incubated with diluted peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan) as recommended by the manufacturer and then developed in 0.05% 3,3′-diaminobenzidine–0.03% H_2O_2 in 0.05 M Tris-HCl (pH 7.4).

Mosquito live feeds. Four-day-old female *A. stephensi* mosquitoes (pre-passive immunization) were starved and then allowed to feed on mice 3 days after the mice were infected with *P. yoelii* (10^6 parasitized cells per mouse). The mice were subsequently injected intraperitoneally or intravenously with MAb, $F(ab')_2$, or Fab. A batch of starved mosquitoes was fed on these mice (post-passive immunization) 1 h or 10 min after the injection. The engorged mosquitoes were selected and maintained at 24°C for 9 days. The significant difference in the median number of oocysts between the pre- and post-passive immunization groups was tested by the Mann-Whitney *U* test. The following nomenclature (8) has been adopted to express the quantitative results from transmission-blocking assays: suppression of oocyst development refers to the ability of the test reagent to suppress the median number of oocysts per midgut to less than 50% of the pre-passive immunization value but not to reduce the ratio of uninfected mosquitoes to total mosquitoes dissected to below 0.5; reduction of transmission refers to the ability of the MAb to reduce the ratio of uninfected mosquitoes to total mosquitoes dissected to below 0.5 but not below 0.1; and complete transmission-blocking activity refers to reduction of the ratio of uninfected mosquitoes to total mosquitoes dissected to below 0.1. For experiments with *P. berghei*, mice were pretreated with phenyl-hydrazine (Wako) at 6 mg/ml in PBS in a single intraperitoneal injection of 200 μl 3 days before infection with *P. berghei* for direct feeds by mosquitoes. Mosquitoes were maintained at 19°C for 12 days after feeding on mice.

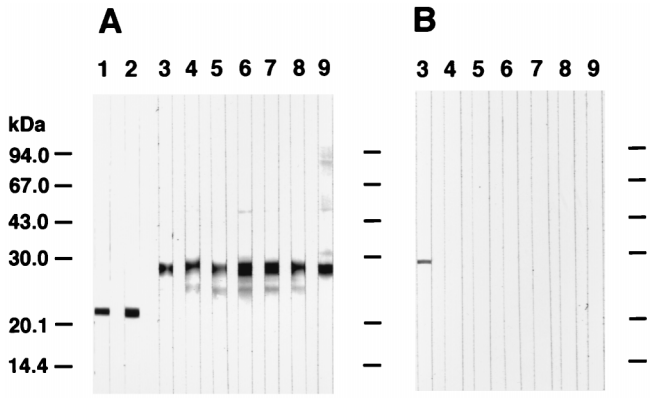

FIG. 1. (A) Reactivity by immunoblotting of anti-Pys21 and anti-Pys25 MAbs to *P. yoelii* ookinetes. (B) Reactivity by immunoblotting of anti-Pys25 MAbs to *P. berghei* ookinetes. SDS extracts of zygotes and ookinetes were size fractionated on an SDS–5 to 20% gradient polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Strips 1 and 2 (panel A) and strips 3 to 9 (both panels) were reacted with MAbs 2, 10, 14, 15, 16, 17, 19, 20 and 4, respectively. Molecular mass markers are indicated.

RESULTS

Characterization of MAbs and their determinants. Nine mouse IgG MAbs (MAbs 2, 4, 10, 14, 15, 16, 17, 19, and 20) that bind the surface of acetone-fixed zygotes and ookinetes of *P. yoelii* and one (MAb 1) that recognizes merozoites of *P. yoelii* were obtained (data not shown). The isotype of all MAbs was IgG1, except for MAb 10 (IgG2a). MAbs 2 and 10 specifically recognized a 21-kDa protein of *P. yoelii* (Pys21), and MAbs 4, 14, 15, 16, 17, 19, and 20 recognized a 28-kDa protein of *P. yoelii* (Pys25), under nonreducing conditions (Fig. 1A). When the antigens were treated with a reducing agent, these nine MAbs no longer reacted with any proteins of *P. yoelii* ookinetes by immunoblotting (data not shown). Furthermore, two (MAbs 14 and 19) of seven anti-Pys25 MAbs were found to cross-react with *P. berghei* ookinetes by IFA (data not shown), and MAb 14 cross-reacted with *P. berghei* ookinetes by Western blotting after the SDS-PAGE under nonreducing conditions. The antigenic molecule recognized by MAb 14 was determined to be a 28-kDa protein of *P. berghei* (Pbs25) by Western blotting (Fig. 1B). MAb 19 had no cross-reaction with *P. berghei* ookinete protein by Western blotting (Fig. 1B). The precise locations of Pys21 or Pys25 were determined by immunoelectron microscopy. With anti-Pys25 MAb 16, the antigenic molecule was seen to be distributed over the surface of the *P. yoelii* mature ookinete (Fig. 2). No detectable immunolabeling of zygotes or ookinetes was seen with control antibody MAb 1 (data not shown).

Effects of MAbs on oocyst formation in vivo. Purified MAbs were tested for transmission-blocking activity in a series of passive immunization experiments (Table 1). Anti-Pys21 MAb 2 or MAb 10 administered intraperitoneally demonstrated the suppression of *P. yoelii* oocyst development by an average of 5 or 12% of the pre-passive immunization value. Furthermore, anti-Pys25 MAbs administered intraperitoneally had complete transmission-blocking activities. Antimerozoite monoclonal antibody MAb 1 had no detectable transmission-blocking activity. To determine whether the anti-Pys25 MAbs have trans-

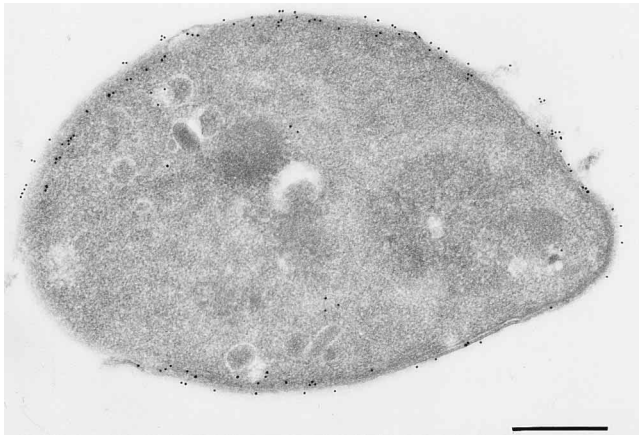

FIG. 2. Distribution of Pys25 on the surface of a *P. yoelii* mature ookinete. Immunogold labeling of Pys25 with MAb 16 showed the distribution of gold particles over the cell surface of a whole mature ookinete. Scale bar = 0.5 μm.

mission-blocking effects on *P. berghei*, purified anti-Pys25 MAbs (MAbs 4, 14, 15, and 19) were tested in a series of passive immunization experiments using *P. berghei*. Only MAb 14 administered intraperitoneally showed a significant reduction of infectivity to mosquitoes (median number of oocysts: pre-passive immunization, 18.0; post-passive immunization, 0). However, MAbs 4, 15, and 19 and control MAb 1 did not elicit any transmission-blocking effect on *P. berghei* (data not shown).

Effects of MAb 2, MAb 4, and their F(ab′)$_2$ and Fab fragments injected in DBA/2 mice on oocyst formation. To assess the contribution of complement to antibody-mediated transmission-blocking activity, we examined the effects of anti-Pys21 MAb 2 and anti-Pys25 MAb 4 injected intravenously into C5-deficient DBA/2 mice on the formation of oocysts of *P. yoelii* in the *A. stephensi* midgut. The median number of oocysts per mosquito midgut fed on the MAb 4-injected mouse expressed as a percentage of that found in pre-passive immunization group was 0.02% at the dosage of 154 μg/mouse. Infectivities to mosquitoes of the passively MAb 2-immunized group were slightly higher than infectivities of mosquitoes of the passively MAb 4-immunized group, 7.3% of the pre-passive immunization value at 360 μg/mouse and 2.3% of the pre-passive immunization value at 720 μg/mouse. Both MAb 4 and MAb 2 had transmission-blocking effects even in the absence of complement. To determine whether the association of antibody-dependent cell-mediated cytotoxicity was present, we made F(ab′)$_2$ fragments of either MAb 2 or MAb 4 and then performed a passive immunization experiment using DBA/2 mice. As shown in Fig. 3, suppression of infectivity to mosquitoes by F(ab′)$_2$ of MAbs 2 and 4 was dose dependent. F(ab′)$_2$ of MAb 4 had a stronger effect on the suppression of oocyst formation than that of MAb 2. Furthermore, we studied the effects of the Fab fragment of each MAb on the infectivity of *P. yoelii* to mosquitoes. The Fab of MAb 4 had an effect on infectivity similar to that of the F(ab′)$_2$ fragment of MAb 4. In contrast, the Fab fragment of MAb 2 did not suppress the infectivity to mosquitoes.

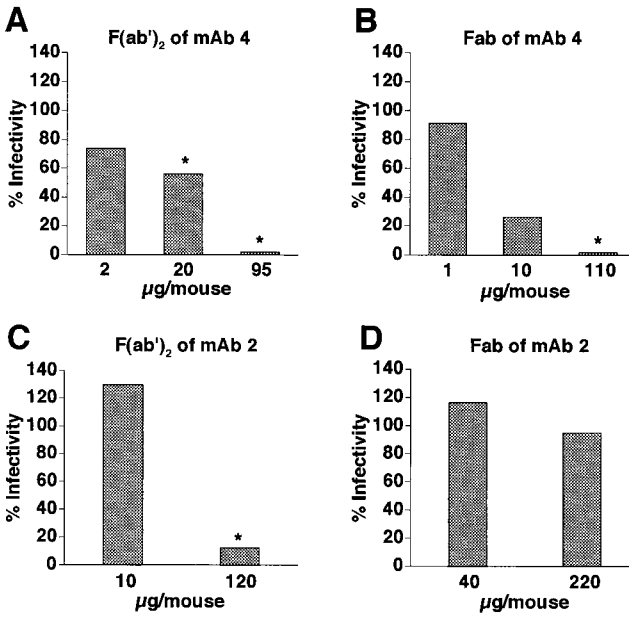

FIG. 3. Effects of the F(ab′)$_2$ and Fab fragments of MAbs 2 and 4 on the infectivity of *P. yoelii* to *A. stephensi*. Each bar represents the median number of oocysts per mosquito midgut expressed as a percentage of that found in the pre-passive immunization group and is derived from the dissection of 20 to 30 pre- or post-passive immunization group of mosquitoes. (A) Transmission-blocking effects of F(ab′)$_2$ fragments of MAb 4; (B) transmission-blocking effects of Fab fragments of MAb 4; (C) transmission-blocking effects of F(ab′)$_2$ fragments of MAb 2; (D) transmission-blocking effects of Fab fragments of MAb 2. Asterisks represent significant statistical differences ($P < 0.05$) in the number of oocysts between the pre- and post-passive immunization groups tested by the Mann-Whitney *U* test.

TABLE 1. Effects of MAbs on infectivity of *P. yoelii* to *A. stephensi*

MAb	Amt of MAb (μg/mouse)	Group of mosquitoes[a]	Median no. of oocysts/midgut (range)	No. of mosquitoes with oocysts/no. dissected	*P* value[b]
1	0	Pre	312.0 (39–639)	38/38	
	690	Post	287.5 (32–641)	40/40	NS
2	0	Pre	394.0 (61–718)	35/35	
	720	Post	20.5 (0–108)	34/38	<0.0001
4	0	Pre	201.0 (39–629)	39/39	
	690	Post	0 (0)	0/40	<0.0001
10	0	Pre	462.0 (61–734)	31/31	
	720	Post	54.0 (0–153)	31/32	<0.0001
14	0	Pre	82.5 (0–623)	29/32	
	750	Post	0 (0)	0/49	<0.0001
15	0	Pre	89.0 (6–306)	43/43	
	780	Post	0 (0)	0/42	<0.0001
16	0	Pre	299.5 (53–612)	40/40	
	750	Post	0 (0)	0/40	<0.0001
17	0	Pre	306.5 (16–596)	40/40	
	750	Post	0 (0)	0/40	<0.0001
19	0	Pre	35.0 (0–673)	32/41	
	774	Post	0 (0)	0/48	<0.0001
20	0	Pre	249.0 (47–567)	39/39	
	759	Post	0 (0)	0/40	<0.0001

[a] Treatment conditions: pre, direct feed of mosquitoes on ICR mice before injection with MAb; post, direct feed on ICR mice after intraperitoneal injection with MAb.

[b] Probability value of statistical analysis of the number of oocysts between pre- and post-passive immunization. NS, differences between the two groups were not statistically significant.

Effects of MAb 2, MAb 4, and their Fab fragments on the development of zygotes to ookinetes in vitro. To determine the effects of MAbs or Fab fragments on the transformation of zygotes to ookinetes in the absence of complement and peripheral blood leukocytes (PBL), we cultivated purified *P. yoelii* gametocytes with each MAb or Fab fragment. MAbs 2 and 4 blocked the formation of ookinetes in vitro. MAbs 2 and 4 appeared to block the in vitro formation of zygotes as well. The

TABLE 2. Effects of MAb 2, MAb 4, and their Fab fragments on the development of zygotes to ookinetes in vitro

Expt no.[a]	MAb or Fab[b]	Concn of MAb (μg/ml)	Median no. of parasites/well (range)	
			Zygotes[c]	Ookinetes[d]
1	None	0	ND[f]	15.5 (10–19)
	MAb 1	345	ND	17.0 (10–21)
	MAb 4	345	ND	0 (0)[e]
2	None	0	30.0 (25–32)	14.5 (11–17)
	MAb 2	320	4.0 (2–5)[e]	2.0 (0–3)[e]
	MAb 4	320	2.0 (1–3)[e]	0.5 (0–2)[e]
	Fab 2	176	25.5 (23–28)	10.5 (10–14)
	Fab 4	167	23.5 (19–25)[e]	4.5 (3–7)[e]
3	None	0	18.5 (17–19)	8.0 (7–12)
	Fab 2	117	20.5 (17–23)	9.5 (9–13)
	Fab 4	111	18.5 (15–21)	6.0 (4–8)
3	None	0	17.5 (16–20)	5.0 (3–6)
	Fab 2	235	19.0 (16–21)	10.0 (9–13)[e]
	Fab 4	223	12.5 (12–16)[e]	1.0 (1–2)[e]

[a] Group of cultures done at the same time.
[b] Treatment conditions: none, cultured with ookinete medium including the same amount of PBS as the experimental group cultured with antibody solution; MAb 1, cultured with MAb 1 as a negative control; MAb 2, cultured with MAb 2; MAb 4, cultured with MAb 4; Fab 2, cultured with Fab fragment of MAb 2; Fab 4, cultured with Fab fragment of MAb 4.
[c] Number of zygotes stained with MAb 4 as the first antibody in 4 μl of cultured parasite suspension on a well of a Multitest slide. Four to six wells were counted in each group.
[d] Number of retort and mature ookinetes stained with MAb 4 as the first antibody in 4 μl of cultured parasite suspension on a well of a Multitest slide. Four to six wells were counted in each group.
[e] Statistically significant compared with the corresponding control (none) group ($P < 0.05$).
[f] ND, not done.

Fab fragment of MAb 4 significantly reduced the number of ookinetes. In contrast, the number of ookinetes that formed in cultures with MAb 1 or the Fab fragment of MAb 2 were equal to control cultures (ookinete medium alone) (Table 2).

DISCUSSION

P. falciparum and *P. gallinaceum* have at least two distinct ookinete surface proteins (7). The antibodies raised against these proteins demonstrated a transmission-blocking effect. However, only Pbs21 from *P. berghei* has been detected for rodent malaria parasites (14). In this study, we have identified two ookinete surface proteins in *P. yoelii* (Pys21 and Pys25), one of which is also present in *P. berghei* (Pbs25), that are target antigens of transmission-blocking antibodies. Accordingly, it became clear that all the *Plasmodium* species, including *P. knowlesi* (4), so far investigated have at least two distinct ookinete surface proteins.

The transmission-blocking activity of MAb 4 is more effective than that of MAb 2 in *P. yoelii*. The transmission-blocking effects of MAbs against the various proteins are also different in *P. gallinaceum*. Anti-Pgs25 MAb blocked transmission effectively more than anti-Pgs28 MAb (5). Although the passive immunization of mice by MAb 13.1 against Pbs21 inhibited the transmission of *P. berghei*, the inhibition was not dose dependent and not complete even at high doses (16). When we passively immunize mice with MAb 14, transmission blockade of *P. berghei* is nearly complete. Therefore, the difference in efficacy between the transmission-blocking MAbs against two distinct ookinete surface proteins is consistent in *Plasmodium* species.

The MAbs of isotype IgG2a against Pfs230, a gamete surface protein of *P. falciparum*, mediated complement-dependent lysis of the gametes and gave almost total suppression of infectivity of gametocytes to mosquitoes. The other MAbs of isotype IgG1 against Pfs230 failed to lyse the gametes in the presence of complement and had no significant transmission-blocking effects (17). In this study, the difference of transmission-blocking activities between MAbs 4 and 2 is not due to immunoglobulin isotype or complement-activating ability. Likewise, complement is not required to develop the transmission-blocking effects of anti-Pfs25 MAbs (22).

With regard to the mechanism of transmission-blocking antibodies, IgG but not Fab from MAb 13.1 reduced ookinete formation by *P. berghei* in culture with PBL. Thus, one mechanism of action of MAb 13.1 is antibody-mediated phagocytosis by PBL or antibody-dependent cell killing (15). Transmission-blocking MAbs to Pfs25 (8) and to Pgs25 (5) do not block zygote-to-ookinete transformation; rather, these antibodies interfere with development that occurs later, sometime between the ookinete's penetration of the peritrophic matrix and midgut epithelium and the parasite's formation of an oocyst (18). In contrast to these results, both MAb 4 and MAb 2 blocked transmission by interfering with transformation of zygotes to ookinetes in the absence of complement and antibody-dependent cell-mediated cytotoxicity. Moreover, MAb 4 might prevent the function of Pys25, which seems to play a significant role in the zygote-to-ookinete transformation.

There is only one study on the coimmunization with two different transmission-blocking malaria vaccines. The combination of antibodies to Pfs28 with those to Pfs25 substantially increases the efficacy of either alone and provides a scientific basis for combining the two antigens in a single transmission-blocking vaccine (2). By using PCR, we identified a new ookinete surface protein gene of *P. berghei*, Pbs25 (20), which was structurally similar to both Pfs25 and Pgs25. Moreover, we have recently cloned two homologous genes of *P. yoelii*, Pys21 and Pys25 (21a). Thus, we named the 21- and 28-kDa ookinete surface proteins of *P. yoelii* Pys21 and Pys25, respectively. The rodent malaria model should be a good system to use for clarifying the mechanisms by which coimmunization with two distinct antigens is more effective than immunization with either antigen alone. In addition to testing the potency of a vaccine candidate in vivo rather than by the ex vivo membrane feeding assay, the murine model has the benefit of access to a variety of genetic mutations in the mammalian host. Defects in complement, coagulation, and T and B cells and the ability to compare immune responses to vaccination in naive and previously infected mice make the murine model a facile system for assessing the contribution of a variety of host factors in diminishing or enhancing transmission of malaria parasites to mosquitoes.

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