

Complement-Mediated Lysis of *Plasmodium falciparum* Gametes by Malaria-Immune Human Sera Is Associated with Antibodies to the Gamete Surface Antigen Pfs230

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Antibodies to the sexual-stage surface antigens of *Plasmodium falciparum*, Pfs230 and Pfs48/45, can abolish the infectivity of gametes to mosquitoes; these antigens have been proposed as candidates for inclusion in a malaria transmission-blocking vaccine. One possible mechanism of antibody-mediated transmission blocking is complement-mediated gamete lysis. We have used a panel of human sera from geographically distinct regions where malaria is endemic to investigate whether this may be a mechanism of naturally acquired transmission-blocking immunity to *P. falciparum*. By immunoprecipitation, we have shown that antibody recognition of Pfs230 and Pfs48/45 is limited, despite universal exposure to *P. falciparum* gametocytes. In vitro complement-mediated lysis of *P. falciparum* gametes was positively associated with the presence of antibodies to Pfs230 but not with antibodies to the N-terminal region of the precursor molecule (Pfs260), which is shed from the gametocyte surface at the time of gametogenesis. Similarly, antibodies to two other gametocyte-specific proteins, Pfs48/45 and Pfg27/25, were not associated with gamete lysis. All sera which mediate gamete lysis contain immunoglobulin G1 (IgG1) and/or IgG3 antibodies to gamete surface proteins as determined by an enzyme-linked immunosorbent assay. These data suggest that Pfs230 is a major target of complement-fixing antibodies which may be important for antibody-mediated transmission-blocking immunity.

Transmission of malaria is dependent upon the successful completion of the sexual phase of the life cycle within the mosquito vector. Interrupting the development of the sexual stages by preventing fertilization or the establishment of zygotes on the mosquito midgut is recognized as a potential strategy for the control of malaria transmission.

Gametocytes, gametes, and zygotes express novel, stage-specific antigens on their surfaces (16, 31), rendering them susceptible to immune attack. Both antibody-mediated and cell-mediated effector mechanisms are believed to play a role in transmission-blocking immunity (28), although most attention has focused on defining the antigenic targets of antibody-dependent mechanisms. The major gametocyte and gamete surface antigens of *Plasmodium falciparum*, Pfs230 and Pfs48/45, which might form the basis of a transmission-blocking vaccine, are known to be targets of transmission-blocking monoclonal antibodies (MAbs) (19, 22, 30). Both proteins are synthesized early in gametocyte development (16). Pfs230 is derived from processing a precursor molecule which has been traditionally termed Pfs260 (19, 32). The predicted size of the precursor gametocyte form is 360 kDa, and that of the mature protein is 310 kDa (34). In this paper, we shall refer to the proteins as Pfs260 and Pfs230. It is believed that at the time of gametogenesis (emergence of the gamete from the host erythrocyte in the mosquito gut), Pfs260 is specifically cleaved, with the membrane-associated Pfs230 fragment remaining on the gamete surface (32, 35), where it exists as a complex with Pfs48/45 (16).

All Pfs230-specific transmission-blocking MAbs studied to

date are directed against conformational epitopes and do not recognize the protein in reduced form (21). Another common feature is that they are all of complement-fixing immunoglobulin G (IgG) subclasses and do not block parasite development unless complement is present (21, 26). Furthermore, immunization of mice with part of Pfs230 produced as a bacterial fusion protein induced antibodies which block transmission in the presence of complement (36). The doublet Pfs48/45 is produced by differential processing of the products of a single gene. Studies using Pfs48/45-specific MAbs have revealed identical epitopes on both components of the doublet (31). Some of these MAbs block infectivity without the requirement for complement, while others are dependent on the presence of complement; some require the presence of other MAbs with synergistic blocking effects (4, 22, 28, 31).

Antibodies against both Pfs230 and Pfs48/45 are found in human sera collected in regions where malaria is endemic (5, 8, 23, 24, 27). Graves et al. (8) demonstrated a correlation between suppression of infectivity of gametocytes to mosquitoes in membrane feeding assays and the presence of antibodies to Pfs230 in sera from Papua New Guinea. However, studies elsewhere have found no significant correlation between infectivity of gametocytes and the presence of antibodies to Pfs230 (18, 25).

Antibodies to the gametocyte and gamete-specific intracellular protein Pfg27/25 are found in the sera of most, if not all, people exposed to gametocytes (23), but these do not appear to play a significant role in transmission-blocking immunity. Although MAbs against Pfg27/25 have been shown to mediate transmission blocking (37), this may be due to a cross-reaction with Pfs230. Pfg27/25 antibodies do, however, serve as a useful marker for exposure to *P. falciparum* gametocytes.

In animal malaria models, several potential antibody-mediated mechanisms of transmission-blocking immunity have been

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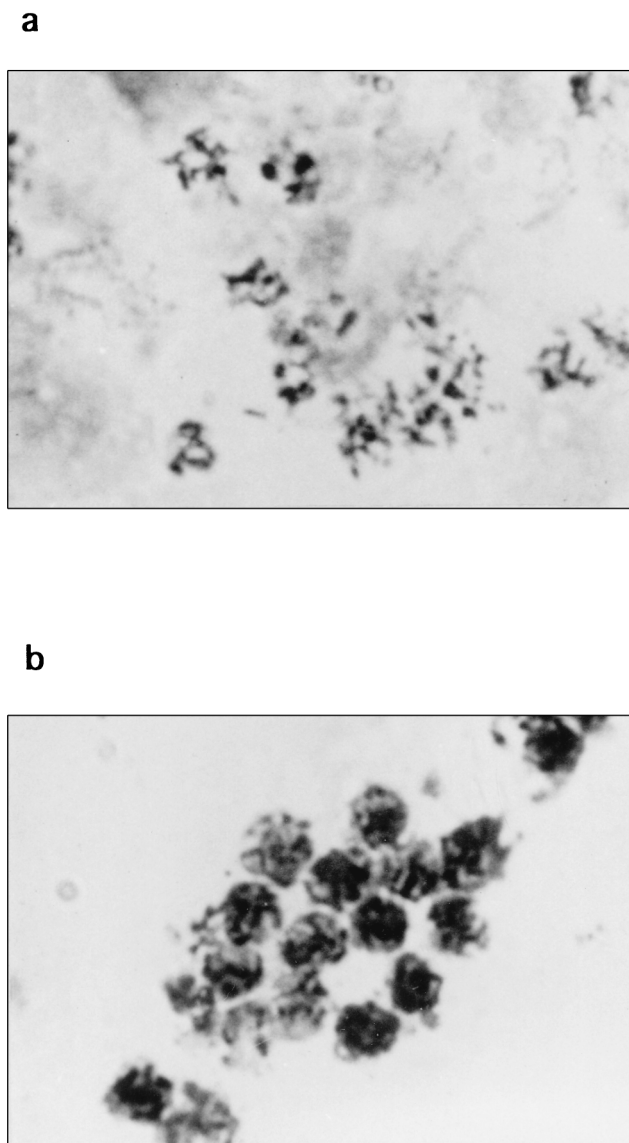


FIG. 1. In vitro lysis of *P. falciparum* gametes in the presence of immune serum and complement (a) or of immune serum and inactivated complement (b).

described, including complement-mediated lysis (14), agglutination of gametes (1), and antibody-dependent phagocytosis (20). The former two mechanisms have also been demonstrated in MAb-mediated transmission-blocking immunity against *P. falciparum* gametes (22). However, the mechanisms by which naturally acquired antibodies to gametocyte and gamete antigens mediate transmission-blocking immunity have not yet been elucidated. In the present study, we have tested human sera from The Gambia and Papua New Guinea for their ability to mediate complement-dependent lysis of macrogametes of *P. falciparum* in vitro. We have also looked, by immunoprecipitation, for antibodies to Pfs260, Pfs230, Pfs48/45, and Pfg27/25 and have determined the IgG subclass of antibodies to gametocyte antigens by an enzyme-linked immunosorbent assay (ELISA). We have evaluated the relationship between the ability of sera to mediate gamete lysis and antibody status and have found that while antibodies to Pfs230 are positively associated with complement-dependent lysis, anti-

bodies to other gametocyte-specific proteins (Pfs48/45, the N-terminal region of the Pfs260 precursor molecule, and Pfg27/25) are not. All sera which mediated gamete lysis contained IgG1 and/or IgG3 antibodies (human IgG subclasses which fix complement in antibody-mediated reactions) to gamete surface antigens, but not all antibody-positive sera were able to mediate lysis, suggesting that the epitope specificity of the antibody, as well as its isotype, may be important for its function.

MATERIALS AND METHODS

Parasites. Gametocytes of *P. falciparum* clone 3D7A (33) were grown in culture as described previously (12), with RPMI 1640 (Gibco, Paisley, Scotland; pH 8.7) supplemented with 10% heat-inactivated normal human serum, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), hypoxanthine, and 5% NaHCO₃ (complete medium).

Fifteen to 17 days after expansion of a culture with fresh erythrocytes, mature gametocytes were harvested and stimulated to undergo gametogenesis for 1 h in complete medium to which an extract of mosquito pupae had been added (17). Gametes were enriched on a discontinuous Nycodenz (Nijegaard, Norway) gradient (30) in M199 medium (Gibco). The gamete fraction was recovered from the interface between 11 and 6% Nycodenz gradients and washed twice in RPMI 1640.

Gametocytes destined for ¹²⁵I labelling of internal antigens were harvested after 14 days in culture and enriched on discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradients (6). Mature gametocytes were isolated from the interface between 30 and 45% Percoll gradients.

Human sera. Sera were collected during the malaria transmission season from 61 villagers, aged between 1 and 77 years, living near the town of Farafenni, situated on the north bank of the Gambia River. We also analyzed sera from 27 adults, collected as part of a longitudinal survey, from the village of Brefet, south of the Gambia River. Malaria is seasonally endemic in all areas of The Gambia (10). Finally, samples collected in Papua New Guinea as part of a cross-sectional malaria survey of individuals over 4 years of age from the villages of Tau, in East Sepik Province, and Agan, in Madang Province, were used (9). Malaria is highly endemic in both of these villages (8) (sera from these areas are termed malaria exposed).

Control sera ($n = 40$) were obtained from nonexposed European donors (nonexposed European sera).

Assay for complement-mediated lysis of gametes. Approximately 2×10^5 gametes of *P. falciparum*, in 10 μ l of M199 medium, were aliquoted into each well of a sterile, round-bottom, 96-well microtiter plate. Ten microliters of 20% fresh, nonimmune human serum in phosphate-buffered saline (PBS) was added to each well as a source of complement. This serum had been absorbed on swollen agarose and preincubated for 30 min with 5×10^5 gametes to remove nonspecific cytotoxic activity. Control wells received 10 μ l of nonimmune human serum which had been heat inactivated at 56°C for 30 min to destroy the complement activity. Test sera (malaria exposed) were heat inactivated at 56°C, and 10 μ l of each serum was then added to paired gamete wells, one which contained active complement and the other which contained heat inactivated complement. The final dilution of test serum was 30%. Plates were incubated at 37°C for 40 min. The contents of each well were pipetted onto single wells of a multispot slide and allowed to dry overnight at room temperature before being stained with Giemsa stain. Slides were then analyzed (blind) by oil immersion microscopy, and results were confirmed by a further blind analysis by another reader. European sera were used as negative controls.

¹²⁵I labelling, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of *P. falciparum* gametocytes. Mature gametocytes were purified on discontinuous Percoll gradients as described above. Approximately 6×10^7 gametocytes were washed twice in incomplete medium and extracted with 50 μ l of NETTI (6) (0.15 M NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4], 0.5% Triton X-100, 0.05% Na azide, and the protease inhibitors [Sigma, Poole, England] phenylmethylsulfonyl fluoride [1 mM], pepstatin [0.1 mM], TPCK [*N*-tosyl-L-phenylalanyl chloromethyl ketone; 0.1 mM], EDTA [1 mM], EGTA [1 mM], and *N*-ethylmaleimide [1 mM]) by vortexing for 1 min and then spinning at top speed in a microcentrifuge for 5 min at 4°C. The extract was labelled with ¹²⁵I by the IODOGEN method as described previously (11).

Labelled gametocyte proteins were made up to 5 ml with NETTI and then stored at 4°C. Twenty-five microliters of gametocyte protein was incubated with 20 μ l of neat, heat-inactivated human serum, 40 μ l of 25% protein G-Sepharose (Pharmacia), and 400 μ l of NETT (NETTI without protease inhibitors). This was incubated on a rotating wheel overnight at 4°C. The beads were washed once with NETT, then with NETTS (NETT with 0.65 M NaCl), and finally with NETT again. After washing, the beads were resuspended in 60 μ l of nonreducing SDS-PAGE sample buffer (62.5 mM Tris, 5% SDS, 10% glycerol, 0.01% bromophenol blue). Samples were incubated at 100°C for 5 min and spun at top speed in a microcentrifuge for 5 min before being loaded onto a 5 to 15% gradient SDS-PAGE gel. Positive control immunoprecipitations were performed with the Pfs230-specific MAb 12F10 (21). Nonexposed European sera showed no

TABLE 1. Proportions of sera from regions where malaria is endemic mediating complement-dependent lysis of *P. falciparum* gametes in vitro and antibody recognition of specific gametocyte or gamete antigens

| Serum source (region and subjects) | No. of sera | | | | |
|---------------------------------------|-------------|-----------------------|-------------------|-----------------------------|-----------|
| | Total | Lysis positive | Antibody positive | | |
| | | | Pfs260/230 | Pfs260 (N terminus only) | Pfs48/45 |
| Farafenni, The Gambia | | | | | |
| Children | 20 | 8 (40.0) ^a | 8 (40.0) | 7 (35.0) | 11 (55.0) |
| Adults | 41 | 18 (43.9) | 19 (46.3) | 17 (41.5) | 24 (58.5) |
| Brefet, The Gambia, Adults | 27 | 13 (48.2) | 15 (55.6) | 8 (29.6) | 21 (77.8) |
| Papua New Guinea | | | | | |
| Children | 8 | 3 (37.5) | 2 (25.0) | 3 (37.5) | 4 (50.0) |
| Adults | 18 | 12 (66.7) | 8 (44.4) | 4 (22.2) | 10 (55.6) |
| Overall total | 114 | 54 (47.4) | 52 (45.6) | 39 (34.2) | 70 (61.4) |

^a Values in parentheses are percentages.

reactivity with Pfg27/25, Pfs260/230, or Pfs48/45 (data not shown), and sera of subjects from regions of endemicity negative for Pfs260/230 and Pfs48/45 are included in all gels.

¹²⁵I labelling of gamete surface antigens. Purified gametes were labelled as described for gametocytes except that detergent extraction was performed after surface proteins were iodinated such that only those proteins on the gamete surface were radiolabelled. Immunoprecipitations were performed as described above, with the additional positive control using MAb 3E12 which is specific to Pfs48/45 (4). Nonexposed European sera showed no reactivity with Pfg27/25, Pfs260/230, or Pfs48/45 (data not shown) and sera of subjects from regions of endemicity negative for Pfs260/230 and Pfs48/45 are included in all gels.

IgG subclass analysis by ELISA. Mature gametocytes, purified on Percoll as described above, were prepared by the methods of Bordier (2), Kumar (15), and Roeffen et al. (25) for enrichment of Pfs230 by extraction of gametocytes in Triton X-114 (1% in 10 mM Tris-HCl buffer [pH 7.4] containing protease inhibitors). The aqueous phase derived from this procedure was used as the antigen source for the ELISA.

Immulon-4 microtiter plates (Dynatech, Billingham, United Kingdom) were coated with 1% poly-L-lysine in PBS (25) and washed six times with PBS-0.05% Tween 20 before the addition of the aqueous gametocyte extract (see above) to give an equivalent of 10⁵ gametocytes per well. Plates were incubated overnight at 4°C, washed six times, and then blocked with 5% skim milk in PBS-Tween 20 at 37°C for 3 h; this was followed by a further six washes. Human sera diluted at 1/200 in 1% skim milk in PBS-Tween 20 (incubation buffer) were added to duplicate wells containing the gametocyte extract and also to control plates containing poly-L-lysine alone. These were incubated for 90 min at 37°C and then washed a further six times. Mouse monoclonal anti-human IgG subclass sera (anti-IgG1, -IgG2, and -IgG4 [Boehringer Mannheim, Lewes, United Kingdom] and anti-IgG3 [Serotec, Oxford, United Kingdom]) were added at the following concentrations in incubation buffer: anti-IgG1 (clone, NL16) at 1/2,000, anti-IgG2 (clone, ROM 1) at 1/250; anti-IgG3 (clone, HP 6050) at 1/2,000, and anti-IgG4 (clone, RJ4) at 1/1,000. These were incubated for 1 h at 37°C and washed six times, a 1/1,000 dilution in incubation buffer of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako, Denmark) was added, and the plates were incubated for 1 h at 37°C. The subclass specificity of these MAbs has been reported previously (13, 29) and was reconfirmed before use. Plates were developed with H₂O₂ as the substrate and *o*-phenylenediamine (Sigma) as the chromogen, and the reaction was stopped after 10 min by the addition of 20 µl of 2 M H₂SO₄ per well. The optical density (OD) was measured at a wavelength of 492 nm. The antigen-specific OD (sOD) was calculated by subtracting the OD of the poly-L-lysine wells from the OD of the gametocyte extract-containing wells. Sera were considered positive for each isotype if the sOD was greater than the normal range (mean + 2 standard deviations) for the 40 nonexposed European sera tested for each subclass.

Statistical analysis. Multiple logistic regression was used to test for an association between lysis and each of the antibody variables. Odds ratios (ORs) and 95% confidence intervals (CI), corrected for potential confounding by age and geographical area, were calculated. All computation was carried out with GLIM 3.77 software.

RESULTS

Complement-mediated lysis. Sera from 114 malaria-exposed individuals were screened for their ability to mediate in vitro

lysis of *P. falciparum* macrogametes in the presence or absence of active human complement. Each serum sample was classed as either positive or negative based on microscopic analysis of Giemsa-stained slides. Lysis was detectable as fragmentation of gametes, with extracellular malaria pigment scattered liberally across the slide. Sera were scored positive if few or no intact gametes were seen and if gametes remained intact in the serum-matched control (in the absence of complement) (Fig. 1a); however, in most cases where lysis occurred, few, if any, intact gametes were found. A serum sample was given a negative score if most gametes remained intact following incubation with active complement (Fig. 1b). Since lysed gametes are detectable only as pigment granules, it was not possible to obtain an exact figure for percentage lysis. Each serum sample was tested on at least two separate occasions, and repeatable results were obtained. No nonspecific cytotoxicity was observed when sera were incubated with gametes and heat-inactivated complement. European control sera were negative in the assay. Table 1 shows the numbers of sera which mediated complement-dependent gamete lysis. Overall, 47% of the sera were positive in the assay (CI, 38.5%, 56.5%). No significant differences were found in the proportions of sera which mediated complement-dependent gamete lysis based on age, sex, or geographical origin of the donor ($P > 0.2$ in all cases).

Immunoprecipitation of total gametocyte antigens. The sera were used to immunoprecipitate radioiodinated proteins from a Triton X-100 aqueous extract of gametocytes (Fig. 2). Immune complexes were absorbed with protein G-Sepharose beads which bind all subclasses of human IgG. Autoradiographs of gels revealed that all sera of subjects from regions of endemicity were positive for antibodies to the gametocyte-specific protein Pfg27/25. Thus, it is likely that all of the individuals tested had been exposed to *P. falciparum* gametocytes. In contrast, recognition of Pfs230 was limited. All sera which immunoprecipitated Pfs230 also recognized Pfs260, the precursor form of the protein (Fig. 2, lanes 6, 7, 9, and 11). Certain sera which were negative for Pfs230 did, however, possess antibodies to Pfs260 (e.g., Fig. 2, lanes 4, 8, and 10). Presumably, these antibodies recognize epitopes within the N-terminal region of the molecule which is cleaved off at gametogenesis. Some sera (e.g., Figure 2, lanes 8 and 10) showed faint bands which appeared to have sizes intermediate between those of Pfs260 and Pfs230; these were recorded as Pfs230 negatives,

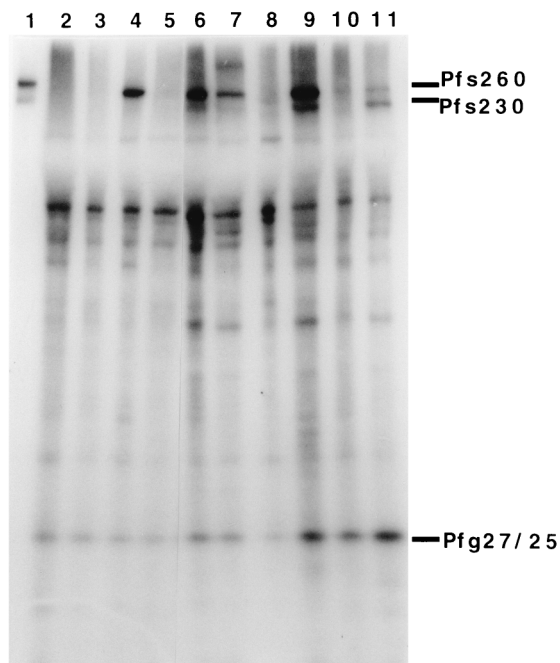


FIG. 2. *P. falciparum* gametocyte proteins extracted with Triton X-100, labelled with ^{125}I , and immunoprecipitated with immune human sera (Brefet, The Gambia). Lanes: 1, MAb 12F10, specific for Pfs230; 2, 3, and 5, Pfs260/230-negative sera; 4, 8, and 10, Pfs260-only-positive sera; 6, 7, 9, and 11, Pfs260/230-positive sera.

and this was confirmed by immunoprecipitation of gamete surface antigens.

Immunoprecipitation of gamete surface antigens. Immunoprecipitations were also performed with ^{125}I -labelled gamete surface antigens extracted with Triton X-100. By use of this method, Pfs48/45 as well as Pfs230 was detectable (Fig. 3). These experiments confirmed the limited recognition of Pfs230 and showed that recognition of Pfs48/45 was also limited. Seropositivity to Pfs230 did not correlate with recognition of Pfs48/45 (e.g., Fig. 3, lanes 3, 10, 12, and 14). Of the 114 serum samples tested, 46% were positive for antibodies to Pfs230 (Table 1). An additional 34% of the sera recognized only the N-terminal region of Pfs260. Sixty-one percent of all sera were positive for anti-Pfs48/45 antibodies. Thirty-three percent (38 of 114) of the sera were positive for antibodies to Pfs260, Pfs230, and Pfs48/45, whereas 12% (14 of 114 sera) were positive for Pfs260 and Pfs230 antibodies but negative for Pfs48/45 antibodies. A further 10% (12 of 114 sera) were positive for anti-Pfs48/45 antibodies but negative for anti-Pfs260/230 antibodies. We found no differences in responsiveness to Pfs230, Pfs260, or Pfs48/45 based on sex, age, or location of the donors.

Relationship between the presence of antigametocyte antibodies and complement-mediated lysis of gametes. Each of the 114 sera were coded for their ability to mediate gamete lysis and whether they were positive for antibodies to Pfs260, Pfs230, and Pfs48/45. All sera were positive for antibodies to Pfg27/25. We were then able to look for associations between the ability to mediate gamete lysis and the presence of each of these antibodies by logistic regression analysis (Table 2). This revealed a highly significant association between gamete lysis and antibodies to Pfs230 ($P < 0.0001$). A serum sample in which anti-Pfs230 antibodies were present was 13 times more likely to mediate gamete lysis than an anti-Pfs230 antibody-negative serum sample, with 73% of sera containing anti-

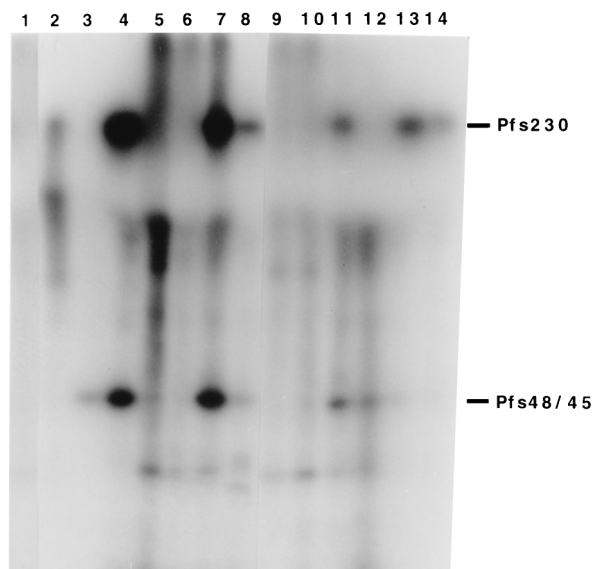


FIG. 3. *P. falciparum* gamete surface proteins labelled with ^{125}I and immunoprecipitated with immune human sera (Farafenni, The Gambia). Lanes: 1, negative control, sera of subjects from regions of endemicity negative for antibodies to Pfs230 and Pfs48/45; 2, MAb 12F10 specific for Pfs230; 3, MAb 3E12 specific for Pfs48/45; 4, 7, 8, and 11, Pfs230- and Pfs48/45-positive sera; 14, Pfs230-positive, Pfs48/45-positive sera; 3, 10, and 12, Pfs230-negative, Pfs48/45-positive sera; 6 and 9, Pfs230-negative, Pfs48/45-sera.

Pfs230 antibodies able to mediate gamete lysis. In contrast, no association was found between antibodies to either Pfs260 or Pfs48/45 and lysis. However, lysis in the absence of antibodies to Pfs230 was detectable in a few sera ($n = 16$); antibodies to Pfs48/45 were detectable in the majority of these sera (9 of 16) but not all, suggesting that antibodies to another antigen may also be mediating lysis. The adjusted OR for the relationship between anti-Pfs230 antibodies and lysis increased substantially after the effects of the other antibodies were accounted for (Table 2), indicating that these other antibodies may have a synergistic effect on the ability of anti-Pfs230 antibodies to mediate lysis. When the regression estimates for lysis in the presence of anti-Pfs230 antibodies are adjusted for age and area, an increase in the OR (Table 2) is again observed, which,

TABLE 2. Association of in vitro complement-mediated gamete lysis and presence of antibodies to Pfs260, Pfs230, and Pfs48/45

| Antibody to: | % (No.) Lysis-positive sera | | OR (95% CI) | P value |
|--------------|-----------------------------|-------------|----------------------------------|---------|
| | Ab ^a positive | Ab negative | | |
| Pfs260 | 48.9 (44) | 41.7 (10) | 1.34 (0.54, 3.33) ^b | 0.53 |
| | | | 1.45 (0.56, 3.74) ^c | 0.71 |
| | | | 0.37 (0.11, 1.2) ^d | 0.10 |
| Pfs230 | 73.1 (38) | 25.8 (16) | 7.81 (3.39, 17.96) ^b | <0.0001 |
| | | | 8.79 (3.65, 21.18) ^c | <0.0001 |
| | | | 12.88 (4.53, 36.64) ^d | <0.0001 |
| Pfs48/45 | 51.4 (36) | 40.9 (18) | 1.53 (0.71, 3.28) ^b | 0.28 |
| | | | 1.56 (0.71, 3.43) ^c | 0.27 |
| | | | 1.05 (0.42, 2.65) ^d | 0.91 |

^a Ab, antibody.

^b Crude OR comparing the odds of lysis between antibody-positive and antibody-negative sera (based on 114 sera).

^c OR adjusted for age and area.

^d OR adjusted for age, area, and the effect of the other two antibodies.

TABLE 3. IgG subclasses of antibodies present within sera which recognize the gametocyte-specific proteins Pfs260, Pfs230, and Pfs48/45

| Antibody to: | No. of antibody-positive sera | | | | | | | | | |
|--------------|-------------------------------|-----------------------|----------|-----------|---------|----------------|-----------|----------|-----------|----------|
| | Lysis positive | | | | | Lysis negative | | | | |
| | Total | IgG1 | IgG2 | IgG3 | IgG4 | Total | IgG1 | IgG2 | IgG3 | IgG4 |
| Pfs260 only | 7 | 6 (85.7) ^a | 0 (0) | 7 (100) | 0 (0) | 27 | 25 (92.6) | 3 (11.1) | 27 (100) | 4 (14.8) |
| Pfs260/230 | 36 | 26 (72.2) | 9 (25) | 29 (80.5) | 2 (5.5) | 13 | 10 (76.9) | 6 (46.2) | 12 (92.3) | 1 (7.7) |
| Pfs48/45 | 32 | 25 (78.1) | 7 (21.9) | 26 (81.2) | 2 (6.2) | 33 | 22 (66.7) | 9 (27.3) | 27 (81.8) | 3 (9.0) |

^a Values in parentheses are percentages.

although not statistically significant, is indicative of a difference between sera from adults and children in their ability to mediate gamete lysis (50% of adult sera mediated lysis compared with 39% of sera from children). In contrast, the OR for Pfs48/45 decreases when adjustments are made for other antibodies, suggesting that any effect of anti-Pfs48/45 antibodies may be due to the simultaneous presence of antibodies against Pfs230.

Gametocyte-specific IgG subclass analysis. The IgG subclass of gametocyte-specific responses was determined for the majority of sera, by use of a Pfs230-enriched gametocyte antigen preparation in an ELISA. Extraction of gametocyte antigens in TX-114 has been shown previously to produce an enriched source of Pfs260/230 (15). This was confirmed by SDS-PAGE analysis. Three major bands were observed when the whole extract was run (molecular sizes, 260, 230, and ~100 kDa), but only Pfs260/230 was immunoprecipitated with immune human serum (data not shown). Pfs260/230 therefore appears to be the major antigen in the extract, although other, less immunogenic proteins were also present. It was apparent that IgG1 and IgG3 were the predominant isotypes in both lysing and nonlysing sera (Table 3); this was the case in both children and adults (data not shown). However, the broad similarity in the isotypes of lysing and nonlysing sera hides some interesting differences. First, IgG2 is more prevalent in children (26%) than in adults (18%), and IgG1 and IgG3 are less prevalent in children (IgG1 was present in 82.4% of adults compared with 48.1% of children). Importantly, all sera which mediated complement-dependent lysis contained IgG1 and/or IgG3 antibodies specific for the Pfs230-enriched gametocyte extract. Table 4 shows a summary of the results obtained from logistic regression analysis, which was used to test for a relationship between IgG subclass and lysis. No associations were found between the isotype of the antigametocyte antibodies present and their ability to mediate gamete lysis; however, the number of IgG2- and IgG4-positive sera was low compared to the number of IgG1- and IgG3-positive sera. Of the 13 non-

lysing sera which were anti-Pfs230 antibody positive and tested in the ELISA, all appeared to contain IgG1 and/or IgG3 antibodies; thus, the lack of complement-fixing antibodies is not an adequate explanation for their failure to mediate lysis. Of the 10 sera which were negative by immunoprecipitation for antibodies to Pfs260, Pfs230, and Pfs48/45, 8 were positive by ELISA, albeit weakly so. It is possible that these sera recognized the other protein components of the gametocyte extract. A potentially interesting, although not statistically significant, finding was that IgG2 was more prevalent in anti-Pfs230/260 antibody-positive, nonlysing sera than in anti-Pfs230/260 antibody-positive, lysing sera (OR = 0.39 [95% CI, 0.08, 1.76], $P = 0.17$) (Table 3).

DISCUSSION

The aims of this study were to test a panel of human sera from various regions where malaria is endemic for their ability to mediate complement-dependent lysis of gametes in vitro, to look for antibodies against *P. falciparum* gametocyte antigens and the dominant surface antigens Pfs230 and Pfs48/45 in particular, and to look for an association between specific antibodies and gamete lysis. When results from the complement assay were compared with those from the immunoprecipitations, it was clear that gamete lysis was strongly associated with the presence of antibodies against Pfs230 but not with antibodies to either Pfg27/25, Pfs48/45, or the N-terminal processing product of Pfs260. Although not statistically significant, minor differences were found in the ability of sera from individuals of different ages, and from different geographical locations, to mediate gamete lysis when anti-Pfs230 antibodies are present. This may represent age-related maturation of the immune response towards effective transmission blocking.

Gamete lysis was not significantly associated with antibodies to Pfs48/45, but it was more likely to occur in the presence of antibodies to both Pfs230 and Pfs48/45 together, perhaps suggesting some functional interactions between the two sets of antibodies.

An ELISA was designed to assess the influence of antibody isotype on gamete lysis. In particular, we were seeking an explanation as to why some sera failed to mediate gamete lysis when they appeared to recognize Pfs230 by immunoprecipitation. There are considerable practical difficulties in obtaining purified Pfs230 to use in immunoassays. The majority of epitopes recognized by transmission-blocking antibodies are dependent on preserving the intact secondary and tertiary structure of the molecule (19, 21), and it has not yet been possible to express the Pfs230 gene in a manner which produces conformationally correct proteins. In this study, we used an extract of gametocyte proteins as the antigen source for the ELISA, which, although enriched for Pfs260/230, also contained other gametocyte proteins. In humans, gamma globulins of subclass IgG1 and IgG3 fix complement, whereas IgG2 and

TABLE 4. Association of in vitro complement-mediated gamete lysis and IgG subclass of gamete-specific antibodies

| IgG subclass | % (No.) Lysis-positive sera | | OR (95% CI) ^b | <i>P</i> value |
|--------------|-----------------------------|-------------|--------------------------|----------------|
| | Ab ^a positive | Ab negative | | |
| 1 | 46.8 (36) | 41.4 (12) | 1.15 (0.44, 2.97) | 0.78 |
| 2 | 47.4 (9) | 44.8 (39) | 1.22 (0.39, 3.77) | 0.73 |
| 3 | 47.7 (41) | 35 (7) | 1.63 (0.55, 4.83) | 0.38 |
| 4 | 33.3 (3) | 46.4 (45) | 0.50 (0.10, 2.38) | 0.39 |
| 1 and/or 3 | 46.5 (46) | 28.6 (2) | 1.83 (0.31, 10.84) | 0.51 |
| 2 and/or 4 | 42.9 (12) | 46.2 (36) | 0.89 (0.35, 2.28) | 0.81 |

^a Ab, antibody.

^b OR is based on 106 sera adjusted for age, area, and the other subclasses, of which 48 (45%) were lysis positive.

IgG4 do not (3). We found that all sera which mediated lysis contained IgG1 and IgG3 antibodies, but a lack of complement-fixing isotypes did not appear to explain the failure of Pfs230-positive sera to mediate gamete lysis since all Pfs230-positive, lysis-negative sera contained gametocyte-specific IgG1 or IgG3. The inability to obtain pure Pfs230 does impose some limitations on the direct comparison of the ELISA results with those obtained by immunoprecipitation and the gamete lysis assay and may explain why some sera which recognized neither Pfs230 nor Pfs48/45 by immunoprecipitation were positive in the ELISA. Also, since most of the Pfs230 was in the precursor form, the detectable IgG1 and IgG3 may be directed towards epitopes in the N-terminal 50 kDa (which is absent from the mature gamete surface protein) and, as such, have no role in mediating gamete lysis. It is also possible that epitopes not exposed in the native proteins may be accessible to antibodies in the ELISA system, where partial degradation of the antigens may occur. Despite these reservations, the ELISA system was suitable for isotyping specific antibodies to gametocyte antigens, with up to 79% of malaria-exposed sera giving OD values above the normal range for nonexposed sera for one or more of the four subclasses tested.

Approximately 12% of the sera (13 of 114) contained antibodies to Pfs230 (by immunoprecipitation) which appeared (by ELISA) to be of complement-fixing isotypes but did not mediate gamete lysis. It is possible that these antibodies recognize epitopes which are not accessible on the gamete surface and are thus unable to bind live gametes. Alternatively, the proximity of the epitope to the plasma membrane of the gamete may be critical in determining whether lysis can occur (36); the epitopes furthest removed from the parasite membrane may bind antibodies, but formation of the membrane attack complex, the ultimate step in the complement cascade, may be inhibited. Such evasion mechanisms exist in bacteria (7), and with a large, complex molecule such as Pfs230, such a scenario is conceivable. Another possible explanation for Pfs230-positive sera failing to lyse gametes is that non-complement-fixing antibodies compete for epitopes with complement-fixing antibodies, resulting in inhibition of transmission-blocking activity (26). In our study, over half of the anti-Pfs230 antibody-positive non-lysing sera contained IgG2 or IgG4 antibodies to gametocyte antigens, suggesting that competition may be an explanation in some cases.

Sixteen of the sera tested were able to lyse gametes in the absence of antibodies to Pfs230, and although most of these were positive for anti-Pfs48/45 antibodies (9 of 16 sera), some appeared to be seronegative for both of these antigens. It is possible that another surface antigen is the target of complement-fixing antibodies in these sera.

Although antibodies to the mature, processed form of Pfs230 are associated with complement-mediated lysis, antibodies to the N-terminal processing product of Pfs260/230 are not. A recent study demonstrated that the 50-kDa N-terminal polypeptide of Pfs260/230 is absent from the surface of extracellular gametes (35). Our data indicate that this may represent an adaptation for the evasion of transmission-blocking immunity, since antibodies to the N-terminal region should not bind to the gamete surface within the mosquito. Given the high proportion of sera which recognize only the 260 form of Pfs230, the role of processing of Pfs260/230 in immune evasion warrants further study.

The results of the present study indicate that naturally acquired antibodies to Pfs230 act to suppress gamete development in a complement-dependent manner and are consistent

with findings of previous studies using MAbs raised against this protein. Studies using mosquito membrane feeding experiments are under way to assess the role of these antibodies in transmission-blocking immunity *in vivo*.

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