Transmission Blockade of *Plasmodium falciparum* Malaria by Anti-Pfs230-Specific Antibodies Is Isotype Dependent

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By use of the parental hybridoma cell line 63F2A2 that produces specific antibodies of immunoglobulin isotype G1 (IgG1; 63F2A2.1) against Pfs230, we attempted to enrich for the synthesis of the downstream switch variant IgG2b and IgG2a monoclonal antibodies (MAbs) of the hybridoma cell line (63F2A2.2b and 63F2A2.2a, respectively). The parental IgG1 did not reduce the *Plasmodium falciparum* transmission in a bioassay irrespective of the presence of complement. MAbs 63F2A2.2b and 63F2A2.2a were effective in reducing the infectivity of *P. falciparum* parasites to *Anopheles gambiae* mosquitoes in membrane-feeding experiments. A transmission reduction of 91% was accomplished by the 63F2A2.2b switch variant, and a reduction of greater than 99% was accomplished by the 63F2A2.2a switch variant, but only in the presence of active human complement. Subsequently, the transmission-reducing effect of MAb 63F2A2.2b or 63F2A2.2a was confirmed in vitro by the rapid lysis of newly formed macrogametes or zygotes in the presence of active complement. MAB 63F2A2.1 did not lyse the newly formed macrogametes or zygotes irrespective of the presence of complement.

Malaria is transmitted from humans to mosquitoes through gametocytes that develop in the blood of infected patients. Gametogenesis of *Plasmodium falciparum* in the *Anopheles* mosquito midgut is accompanied by the emergence of the gametocyte from the erythrocyte. From that moment, the extracellular parasites become susceptible to immune factors, such as antibodies and complement, taken up during the blood meal from the vertebrate host (4).

Gametocytes of *P. falciparum* synthesize molecules with apparent molecular sizes of 230 and of 48 and 45 kDa (Pfs230 and Pfs48/45) after extraction with sodium dodecyl sulfate (SDS) and separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Both molecules are expressed on the surface of macrogametes or zygotes. Several monoclonal antibodies (MAbs) reactive to these sexual-stage molecules block transmission of the parasites from the vertebrate host to the mosquito vector. Some of these MAbs suppress the infectivity of malaria gametocytes for mosquitoes in the presence of active human complement (7, 15, 18, 20). To answer the question whether MAb 63F2A2-mediated transmission blockade is isotype or epitope dependent, a number of isotype switch variants were prepared and selected for differences in their capacity to fix complement.

Downstream isotype switch variants occur spontaneously in hybridoma cell lines, with identical binding sites differing only in the heavy-chain isotype (9, 10). The frequency of spontaneously occurring isotype switch variants ranges from $10^{-6}$ to $10^{-5}$ per cell per generation (9, 16), and a sensitive assay is needed to detect the switched immunoglobulin isotype amid a large amount of immunoglobulin of the parental isotype. The technique of sequential sublining combined with screening by isotype-specific enzyme-linked immunosorbent assay (ELISA) has been proven before to be successful (2, 10).

In this study, the importance of the immunoglobulin G (IgG) subclass for transmission-blocking activity is further analyzed by production and testing of a number of switch variants of the anti-Pfs230-specific MAB 63F2A2.

**MATERIALS AND METHODS**

**Parasites and Pfs230 extract.** Mature gametocytes of *P. falciparum* (isolate NF54) were produced in an automated large-scale culture system as described by Ponnudurai et al. (11). Gametocyte isolation and Triton X-114 extraction of Pfs230 was obtained as described previously (20). The freeze-dried aqueous-phase extract was diluted in phosphate-buffered saline (PBS) and used as the antigen in both the competition and the one-site Pfs230 ELISA.

**Antibodies.** (i) MAbs. The anti-Pfs230 MAbs 18F25, 63F2A2 (63F2A2.1), and 63F2A2.1, and 63F3C8 (all isotype IgG1) have been described previously (20, 22). MAB 18F25 was labelled with horseradish peroxidase by the periodate method described by Wilson and Nakanishi (24). The labelled MAb was dialyzed against PBS-thimerosal (0.01%), and fetal calf serum (1%) was added and samples were stored at 4°C. MAbs 32F81 and 18F25 were labelled with fluorescein isothiocyanate (FITC) by the method described by Godding (5). Rat MAbs against different mouse immunoglobulin isotypes, i.e., IgG1, IgG2a, and IgG2b, and horseradish peroxidase-labelled monoclonal rat anti-mouse kappa light-chain antibodies were purchased from CLB (Amsterdam, The Netherlands).

(ii) Complement. Type AB blood from Dutch blood bank donors with no previous malaria exposure was allowed to clot at room temperature for 1 h, and aliquots of the pooled serum were stored at $-70^\circ$ C until required. Freshly thawed samples were used only once as a source of complement. The serum was heated at 56°C for 30 min to inactivate complement activity. The serum was also used as a malaria-negative control serum in the transmission and parasite lysis tests.

Selection, enrichment, and purification of isotype switch variants. Selection and enrichment of isotype switch variants were conducted by the technique of sequential sublining (2, 10). Briefly, the parental 63F2A2.1 hybridoma cells were grown in a 96-well microtitre plate (NUNC; Intermed), containing 1,000 cells per well, in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and 2.5 U of interleukin 6 per ml and cultured in a humidified 5% CO$_2$-in-air incubator at 37°C. After 7 days, the culture supernatants were tested in the isotype-specific ELISA and cells from positive wells were redistributed at 50 cells per well. After a culture period of 4 days, the supernatants were tested, and cells of positive wells were subcultured at 2 cells per well and cloned by limiting dilution. Supernatants were tested for the presence of the parental isotype and/or switch variants. Selected cloned lines were expanded in culture, and ascitic fluids were produced from 10$^6$ hybridoma cells injected in pristane-primed BALB/c mice. IgG was purified from ascitic fluid with a semiautomated fast-performance liquid
chromatography system (Pharmacia) equipped with a protein A column (Pharmacia) as described previously (20).

ELISAs. (i) Isotype-specific ELISA. The isotype-specific ELISA was used as described previously by Boot et al. (2). The reliable ELISA detection limits were 5 ng/ml for IgGl, IgG2b, and IgG2a with standard IgGl isotype controls. Possible cross-reactivity between isotypes was below 0.1%.

(ii) Specific one-site ELISA. A specific one-site ELISA for detection of antibodies against Pf230 was carried out as described previously (20).

All incubation steps were done at room temperature, except overnight incubations, which were done at 4°C. Between the different steps, plates were washed three times with PBS. The ELISAs were performed in duplicate, and the mean value of the optical densities was used for analysis.

Transmission blocking assay. The effect of MAbs on the infectivity of P. falciparum for Anopheles gambiae mosquitoes was tested in a bioassay using membrane feeders as described previously (12, 13, 22). Briefly, while the temperature was maintained at 37°C, 16-day-old cultures containing fertile gametocytes were mixed with prewarmed unfixederythrocytes and Mabs, introduced into prewarmed feeders, and fed to A. gambiae mosquitoes. All Mabs were tested separately in the feeder assay in the presence of active or inactivated human complement. Fully engorged mosquitoes were separated and held at 26°C. Mosquitoes were dissected 7 days after feeding. The number of ooocysts in each mosquito midgut was counted, and William's mean (adjusted geometric mean [GM]) ooocyst number was calculated (23). The adjusted GM was used to determine the reduction activity (20), and values greater than 85% were consistent in subsequent experiments.

Suspension immunofluorescence assay (SIFA). Molecules with an apparent molecular size of 25 kDa (Pf25) appear on the surface of macrogametocytes or zygotes some hours after zygote formation, and this occurrence was used as a marker of the further development of sexual stages in vitro or in the mosquito midgut (feeder assay; see above).

Suspensions of 10⁶ mature gametocytes in 100 μl of RPMI 1640 medium were mixed with 100 μl of malaria-negative serum, 30 μl of active or inactivated complement serum, and 10 μl of a toxoid MAb 63F2A2.2 or the respective switch variants (5 or 25 μg in PBS) and incubated at 27°C for 3 h. Subsequently, 25 μl of the suspension containing 10⁵ parasites was added to 25 μl of FITC-labelled MAb 32F8B1 diluted 1:40 in 0.05% Evans blue in PBS (pH 7.2)−2% 0.1 M glucose−0.1% 2.7 mM Na₂EDTA−0.05% sodium azide (PBG). The parasites were incubated for 1 min, washed with phosphate-buffered glucose solution (i.e., PBG) containing 5% bovine serum (PBGB). PBGB, centrifuged at 16,000 × g for 10 s, and resuspended in 30 μl of PBGB, and placed in a Bärker-Türk counting chamber, and Pf25-positive macrogametocytes or zygotes were counted under a Leitz Ortholux fluorescence microscope (×500 magnification).

Isotype-dependent lysis of macrogametes or zygotes. A 100-μl suspension containing 10⁵ virobl mature gametocytes in RPMI 1640 medium was mixed with 100 μl of malaria-negative serum and incubated at 27°C for 30 min. Twenty-five microliters of this suspension was incubated with 25 μl of FITC-labelled MAb 18F25 for 20 min, and washed with PBGB by centrifugation at 16,000 × g for 10 s, and the pellet was resuspended in 30 μl of PBGB. To this sample, 5 μl (1 μg) of the MAb 63F2A2 switch variant together with 4 μl of serum as a source of a cellular lytic complement was added. Parasites were examined and scored as intact or lysed macrogametocytes or zygotes by both fluorescence and phase-contrast light microscopes (×500 magnification).

RESULTS

Isolation and characterization of switch variants. When seeded at 1,000 cells per well, IgG2b switch variants were detectable from the parental 63F2A2.1 hybridoma after 7 days of culture in one to two wells per microtiter plate. Further cloning and subcloning resulted in the isolation of a 63F2A2 switch variant of the IgGl subclass (63F2A2.2a). By use of a similar protocol, 63F2A2 Mabs of the IgG2a isotype (63F2A2.2a) were obtained from the 63F2A2.2b-producing hybridoma. All switch variants were subjected to limiting dilution conditions to reach purity at the clonal level. The switch variants reacted only in the ELISA that was specific for the particular isotype (data not shown). Figure 1 shows similar binding profiles of the parental MAb 63F2A2.1 and its IgG2b and IgG2a switch variants in the Pf230 one-site ELISA.

Transmission-blocking capacity of isotypes. The effect of IgG subclass specificity on transmission was analyzed in the bioassay in the presence of active or inactivated human complement. In the presence of active complement, anti-Pf230 Mabs 63F2A2.2b and 63F2A2.2a suppressed infectivity of P. falciparum for mosquitoes by ≥90% and ≥99%, respectively, at concentrations of 10 μg per feeder (Table 1). In the presence of inactivated complement, the different isotypes of MAb 63F2A2 did not reduce oocyst number, irrespective of the presence of complement. Oocyst numbers of the control group with active complement were not significantly different from those in the data of the inactivated complement group (by paired-samples t test, t = 0.56, degrees of freedom [df] = 28, and P = 0.58).

Table 2 shows the dose-dependent reduction of oocyst numbers in the presence of MAb 63F2A2.2b or 63F2A2.2a at concentrations ranging from 0.2 to 25 μg per feeder (0.8 to 100 μg/ml). Even at a concentration of 0.2 μg of MAb 63F2A2.2a per feeder, the GM number of oocysts was 0.9 with a standard deviation (SD) of 0.3 in the presence of active complement, which results in a reduction activity of 80%. In several independent experiments, the reduction capacity of MAb 63F2A2.2b was lower than that of MAb 63F2A2.2a.

Twenty-five anti-Pf230 Mabs of IgGl, IgG2b, or IgG2a in the form of heat-inactivated mouse ascites (final dilution, 1:27), provided by R. Carter (17) or developed by one of us

<table>
<thead>
<tr>
<th>MAb 63F2A2 isotype</th>
<th>Complement</th>
<th>Oocyst count a</th>
<th>Expt 1</th>
<th>Expt 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GMW</td>
<td>SD</td>
<td>R/P</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>24.3</td>
<td>5.9</td>
<td>24.5</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>20.7</td>
<td>3.6</td>
<td>35.7</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>3.2</td>
<td>0.7</td>
<td>90.1</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>21.7</td>
<td>4.5</td>
<td>32.6</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>0.2</td>
<td>0.1</td>
<td>99.4</td>
</tr>
<tr>
<td>6</td>
<td>−</td>
<td>27.2</td>
<td>3.3</td>
<td>25.6</td>
</tr>
<tr>
<td>Control b</td>
<td>32.2</td>
<td>6.5</td>
<td>58/59</td>
<td>2.8</td>
</tr>
</tbody>
</table>

a Active (+) or inactivated (−) human complement.

b The data of experiment 2 are the combined results of two independent tests.

c Control is the mean of three feeders per experiment.
TABLE 2. Infectivity of *P. falciparum* gametocytes for mosquitoes in the presence of anti-Pfs230 MAB 63F2A2.2b or 63F2A2.2a at various concentrations with active or inactivated complement.

<table>
<thead>
<tr>
<th>MAB conc (μg/feeder)</th>
<th>Complement</th>
<th>Oocyst count in presence of:</th>
<th>GMW</th>
<th>SD</th>
<th>R</th>
<th>P/D</th>
<th>GMW</th>
<th>SD</th>
<th>R</th>
<th>P/D</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 +</td>
<td>MAB 63F2A2.2b</td>
<td>3.2</td>
<td>0.7</td>
<td>90.1</td>
<td>18/20</td>
<td>0</td>
<td>100</td>
<td>0/20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 -</td>
<td>MAB 63F2A2.2a</td>
<td>20.7</td>
<td>3.6</td>
<td>35.7</td>
<td>20/20</td>
<td>4.3</td>
<td>1.1</td>
<td>4.9</td>
<td>18/20</td>
<td></td>
</tr>
<tr>
<td>5 +</td>
<td>MAB 63F2A2.2b</td>
<td>3.0</td>
<td>0.7</td>
<td>90.7</td>
<td>17/20</td>
<td>0.1</td>
<td>100</td>
<td>0/20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 -</td>
<td>MAB 63F2A2.2a</td>
<td>24.2</td>
<td>4.3</td>
<td>24.5</td>
<td>21/21</td>
<td>3.3</td>
<td>1.0</td>
<td>27.0</td>
<td>16/20</td>
<td></td>
</tr>
<tr>
<td>1 +</td>
<td>MAB 63F2A2.2b</td>
<td>9.5</td>
<td>2.1</td>
<td>70.5</td>
<td>19/20</td>
<td>0.1</td>
<td>100</td>
<td>0/20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 -</td>
<td>MAB 63F2A2.2a</td>
<td>27.3</td>
<td>6.1</td>
<td>15.2</td>
<td>20/20</td>
<td>4.5</td>
<td>1.3</td>
<td>0.4</td>
<td>17/20</td>
<td></td>
</tr>
<tr>
<td>0.2 +</td>
<td>MAB 63F2A2.2b</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.9</td>
<td>0.3</td>
<td>80.1</td>
<td>12/20</td>
<td></td>
</tr>
<tr>
<td>0.2 -</td>
<td>MAB 63F2A2.2a</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.7</td>
<td>1.6</td>
<td>0</td>
<td>16/20</td>
<td></td>
</tr>
<tr>
<td>Control^a</td>
<td>MAB 63F2A2.2b</td>
<td>32.2</td>
<td>6.5</td>
<td>58/59</td>
<td>4.5</td>
<td>1.3</td>
<td>46/59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control^b</td>
<td>MAB 63F2A2.2a</td>
<td>32.2</td>
<td>6.5</td>
<td>58/59</td>
<td>4.5</td>
<td>1.3</td>
<td>46/59</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Active (+) or inactivated (−) human complement.
^b See Table 1, footnote b, for explanation of the different boxheads.
^c ND, not done.
^d Control is the mean of three feeders per experiment.

Table 2 shows the infectivity of *P. falciparum* gametocytes for mosquitoes at various concentrations with active or inactivated complement.

*SIFA and complement-mediated lysis of macrogametes or zygotes.* The mechanism of complement-dependent transmission-blocking activity of MABs 63F2A2.2b and 63F2A2.2a was further analyzed in vitro with activated gametocytes and a SIFA based on antibody reactivity to Pfs25, thereby monitoring development of macrogametes or zygotes 3 h after activation. The actual numbers of macrogametes or zygotes differ in several parasite batches, which makes direct comparison of data of independent experiments impossible. In a representative experiment (Fig. 2), no statistically significant differences were found in macrogametes or zygote numbers between the different isotypes and the controls (no MAB added) when activated complement was added (e.g., by paired-sample t test between IgG2a and control, t = 1.37, df = 2, and P = 0.305). In addition, no significant difference was found between the numbers of macrogametes or zygotes for the control groups (absence of specific anti-Pfs230 MAB) with active complement in comparison with those of groups with inactivated complement (t = 4.13, df = 2, P = 0.054). When the data of the control and the IgG1 groups are compared with respect to the effect of complement, a significant reduction was observed in the presence of active complement (t = 4.43, df = 5, P = 0.007). Active complement in the presence of MAB 63F2A2.1 had no effect, but in the presence of Mac 63F2A2.2b or 63F2A2.2a, the number of macrogametes or zygotes was reduced substantially and almost completely in comparison with the effect of the control group (t = 6.62, df = 2, and P = 0.022 versus t = 6.47, df = 2, and P = 0.023, respectively). Lysis of macrogametes or zygotes by the complement-fixing MAB 63F2A2.2a in the presence of active or inactivated complement is shown in fluorescent micrographs using SIFA and the FITC-labelled anti-Pfs230 MAB 18F25 (Fig. 3). In the absence of lysis, fluorescence was clearly visible at the circumference of apparently intact parasites (Fig. 3A), whereas fluorescence after lysis revealed either no round forms or damaged

![Image A](https://example.com/imageA.png)

**FIG. 3.** Complement-mediated lysis of early *P. falciparum* macrogametes or zygotes in the presence of MAB 63F2A2.2a as observed after staining with FITC-labelled MAB 18F25. The macrogametes or zygotes remained visually intact when incubated with the IgG2a switch variant in the presence of inactivated complement (A) and were lysed in the presence of the IgG2a switch variant and active human complement (B).
round forms with fluorescent debris or fluorescence of incomplete round forms (Fig. 3B). With phase-contrast light microscopy, damaged parasites with extrusion of the pigment or localisation of aggregated pigment at peripheral sites and loss of pigment motility were manifested.

**DISCUSSION**

In the present study, downstream IgG2b and IgG2a switch variant antibodies specific for Pf230 were generated from the parent MAb, 63F2A2.1. In contrast to 63F2A2.1, both 63F2A2.2b and 63F2A2.2a were able to block the transmission of *P. falciparum* to *A. gambiae* mosquitoes in membrane-feeding experiments but only in the presence of active complement. Results from the one-site Pf230 ELISA (Fig. 1) show that the dose-dependent bindings of MAbs 63F2A2.1, 63F2A2.2b, and 63F2A2.2a are comparable. SIFA results suggest a complement-dependent disturbance of the sexual development of macrogametocytes or zygotes after binding of 63F2A2.2b or 63F2A2.2a switch variant MAbs. Even in the presence of active complement, parental MAb 63F2A2.1 did not prevent the development of parasites after activation of the mature gametocytes; however, irrespective of the presence of these MAbs, active complement shows a direct effect on macrogametocytes or zygote development (Fig. 2). This may be explained by an alternative activation of the complement cascade due to a direct interaction between C3 and the parasites similar to the phenomenon applies to other epitopes of Pf230 (20) remains to be determined.

All anti-Pfs230 MAbs of isotype IgG2b or IgG2a tested to date were effective in blocking transmission but only when complement was added to the feeder, whereas IgG1 isotype MAbs were not. To date, there is an absolute correlation between Pf230-related transmission blockade by MAbs, the ability of the isotype to fix complement, and the need for active complement to show transmission blockade by Pf230-binding antibodies. Quakyi et al. (15) already described two Pf230-specific MAbs (both IgG2a) that suppressed infectivity of *P. falciparum* to *Anopheles freeborni* mosquitoes by a complement-mediated lysis of early macrogametes or zygotes. A comparable effect of complement-fixing MAbs of the IgG2a isotype against a *Plasmodium gallinaceum* protein that is analogous to Pf230 was described by Kaushal et al. (7). Confirming our observations, Read et al. (17) also found in a recent study the complement-dependent suppression of transmission of gametocytes of *P. falciparum* 3D7 to mosquitoes with anti-Pfs230 MAbs of isotype IgG2a.

Generally, mouse IgG1 is known to be a poor binder of human complement factor Clq, whereas IgG2b and IgG2a bind Clq effectively. Binding of Clq is the first step in activation of the classical complement cascade, leading to the formation of the membrane attack complex. Mouse IgG2a is more effective than IgG2b in fixation of human complement factors such as Clq, C4b, and C3b (3, 8). Although complement fixation in binding tests is not directly proportional to complement activation, haemolysis experiments suggest that the fixation of C3b is directly proportional to activation of C3b and the terminal lytic sequence (3). The capacity of different murine isotypes to fix complement fits perfectly the ability of MAb 63F2A2 switch variants to interfere with complement.

These results may have important implications for the study of anti-Pfs230 antibodies that are present in 40 to 85% of the endemic human sera (6, 19). The presence of specific anti-Pfs230 antibodies in field sera has been associated with transmission-blocking activity by some investigators (6) but could not be confirmed by others (14) or in our study (20). Immunoprecipitation and ELISA (6, 20) are based on epitope recognition by antibody being independent of the isotype, while a Pfs230-based transmission blockade may be accomplished by an isotype-dependent effector mechanism. When Pfs230 components are used in the design of a transmission-blocking vaccine (15), these findings support the need for an immunization strategy (1, 21) which should induce antibodies of a complement-binding isotype.

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