

# Immune-mediated Thrombocytopenia of Malaria

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**ABSTRACT** Thrombocytopenia frequently complicates malarial infections but the mechanism has not been elucidated. We studied 28 patients with malarial infections and noted that 16 of 17 thrombocytopenic patients had elevated levels of platelet-associated IgG (PAIgG). In all thrombocytopenic patients studied, the level of PAlG returned to normal as the platelet count rose to normal levels. To study the mechanism of the elevated platelet-bound IgG, IgG and F(ab')<sub>2</sub> from patients with recurrent *Plasmodium falciparum* infections was purified and radiolabeled. Labeled and unlabeled *P. falciparum* antigen was also prepared. IgG did not nonspecifically bind to malaria-damaged platelets. Binding studies with <sup>3</sup>H-malarial antigen demonstrated platelets have saturable binding sites for malarial antigen. Increasing concentrations of malarial antigen displaced the <sup>125</sup>I-IgG antimalarial antibody from the platelets. The binding of <sup>125</sup>I-IgG and <sup>125</sup>I-F(ab')<sub>2</sub> was similar and this excluded significant immune complex binding. The thrombocytopenia that complicates at least some malarial infections is caused by immune mechanisms; specific IgG binds to platelet-bound malaria antigen through the Fab portion of the immunoglobulin molecule.

## INTRODUCTION

Malaria is a common protozoan disease transmitted to humans by the Anopheles mosquito. Certain species

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<sup>1</sup> Abbreviations used in this paper: APTT, activated partial thromboplastin time; DIC, disseminated intravascular coagulation; IRMA, immunoradiometric assay; PAlG, platelet-associated IgG.

of *Plasmodia* infect human erythrocytes and the growth and replication of the parasites leads to the rupture of the erythrocytes and the release of parasites into the circulation. Most patients develop malarial-specific antibodies after infection and the binding of antibodies to *Plasmodium* antigens results in circulating immune complexes (1).

Malaria can cause hemostatic abnormalities that range from asymptomatic thrombocytopenia to fulminant disseminated intravascular coagulation (DIC),<sup>1</sup> (2-4). Early investigators suggested that the major coagulation abnormality of malaria was DIC, but in recent years clinicians have recognized that thrombocytopenia is a common and early sign of malaria infections, whereas DIC is rare (3-5). It has been estimated that ~80% of patients infected with either *P. vivax* or *P. falciparum* malaria develop thrombocytopenia during their infection, and although the thrombocytopenia is caused by an increased platelet destruction, the mechanism hitherto has been unknown (4-8). In this report we describe studies investigating the mechanism responsible for the thrombocytopenia of malaria.

## METHODS

### *Patient studies*

**Patient population.** Patients were considered to have malarial infections if they fulfilled the following criteria: (a) they had symptoms compatible with malaria infection including fever; (b) they had a history of exposure; and (c) intraerythrocyte parasites were observed on blood films.

Pooled convalescent patient sera were obtained from patients who had had recurrent *P. falciparum* infections. Control sera were obtained from healthy individuals who had never had malaria.

**Measurement of platelet-associated IgG (PAlG).** PAlG was measured on washed platelets collected from patients with malaria infections using the antiglobulin consumption assay of Dixon and Rosse with the modifications we have described (9-11). The in vitro studies were performed using an immunoradiometric assay (IRMA) (12). The principle of the IRMA is similar to the antiglobulin consumption assay

except that the binding of  $^{125}\text{I}$ -anti-IgG to IgG-coated beads is used as the end point of the IRMA assay, whereas the lysis of IgG-coated sheep erythrocytes is the end point of the antiglobulin consumption assay.

**Coagulation assay.** The prothrombin time, activated partial thromboplastin time (APTT), thrombin clotting time, protamine sulfate paracoagulation assay, and platelet counts on the patient samples were performed using standard techniques. Fibrinopeptide A was measured using a radioimmunoassay (ImCo Corp., Stockholm, Sweden).

### *In vitro studies*

**Preparation of *P. falciparum* antigen.** *P. falciparum* (FCR<sub>3</sub>TC strain) was raised in continuous culture as has been described (13). Human erythrocytes infected with *P. falciparum* were incubated with washed human erythrocytes in RPMI 1640 medium supplemented with 5% pooled human serum and [ $^3\text{H}$ ]isoleucine (New England Nuclear, Boston, MA), 10  $\mu\text{Ci}/\text{ml}$ . After a 48–96 h incubation in candle jars, the erythrocytes were removed, washed, and the malarial antigen disrupted by repetitive freeze-thawing followed by sonication. The supernatant fluid was removed following ultracentrifugation at 35,000  $g \times 120$  min and passed across a 10  $\times$  25-cm Sephadex G-10 column (Pharmacia Fine Chemicals, Lachine, Quebec). The effluent containing the malaria antigen was collected and frozen before use. Unlabeled antigen was prepared identically except that no [ $^3\text{H}$ ]isoleucine was added to the culture. Control erythrocytes were prepared by omitting infected erythrocytes from the culture. These control cells were handled identically to the test erythrocytes, including the addition of [ $^3\text{H}$ ]isoleucine.

**Preparation of monomeric IgG and  $F(ab)_2$  fragments.** Monomeric IgG was prepared by passage of convalescent patient serum or control serum across a 2.6  $\times$  40-cm DEAE-column (Pharmacia Fine Chemicals). The IgG-rich fraction was further purified by gel filtration across a 2.6  $\times$  40 cm S-300 column, (Pharmacia Fine Chemicals).  $F(ab)_2$  was prepared from the IgG fractions by pepsin digestion followed by gel filtration across a 2.6  $\times$  40-cm S-200 column (13). The purity of the IgG and  $F(ab)_2$  was confirmed by Ouchterlony double diffusion immunoprecipitation and analytical polyacrylamide gel electrophoresis in SDS. Before use the preparations were ultracentrifuged (35,000  $g \times 120$  min) to remove aggregates.

The intact and digested immunoglobulins were radiolabeled with  $^{125}\text{I}$ iodine using lactoperoxidase Enzymobeads (Bio-Rad Laboratories Richmond, CA). After labeling, the unbound  $^{125}\text{I}$ iodine was separated from the  $^{125}\text{I}$ -immunoglobulin by gel filtration across a 1  $\times$  25-cm Sephadex G-75 column. Protein determinations were made using the Bio-Rad technique.

**Measurement of malaria-dependent binding of IgG to platelets.** Malaria-dependent binding of IgG to control platelets was determined after incubating patient or control serum with control group O platelets in the presence and absence of unlabeled malarial antigen. The platelets were washed three times and the serum-platelet bindable IgG measured (14), using the IRMA assay (12).

**Measurement of the binding of malarial antigens to platelets.** Increasing concentrations of  $^3\text{H}$ -malarial antigen suspended in 200  $\mu\text{l}$  of 0.15 M phosphate-buffered saline, pH 7.4 (PBS) were incubated with 200  $\mu\text{l}$  washed platelets ( $3.0 \times 10^7$  final) for 60 min at 22°C. Platelet-bound malarial antigen was separated from unbound antigen by passage across an oil barrier. 200  $\mu\text{l}$  of the platelet-antigen mixture was layered onto 200  $\mu\text{l}$  of silicone oil (Dow Corning Corp.,

Midland, MI, 3.3 parts No. 550: 6.7 parts No. 556) and centrifuged at 13,000  $g$  for 7 min. The platelet pellet was recovered and dissolved in 0.5 ml NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL), and added to 10 ml of scintillation fluid (Aqueous Counting Scintillant, Amersham Corp.) for counting in a Beta counter (LKB Instruments, Stockholm, Sweden). Experiments using  $^{51}\text{Cr}$ -labeled platelets demonstrated that >95% of platelets centrifuged through the silicone oil. In the absence of platelets, malarial antigen alone or with patient serum did not cross the oil. In other experiments 100  $\mu\text{g}$  of labeled malarial antigen was incubated with a 100-fold excess of unlabeled malarial antigen and the platelet binding of labeled antigen measured.

**Effect of increasing concentrations of malarial antigen on the binding of  $^{125}\text{I}$ -IgG to platelets.** Increasing concentrations of unlabeled malarial antigen (3–70  $\mu\text{g}$ ) were incubated with 200  $\mu\text{l}$  of patient or control  $^{125}\text{I}$ -IgG (60  $\mu\text{g}$ ) for 30 min at 22°C. 200  $\mu\text{l}$  of platelets ( $3.0 \times 10^7$  final) were added and the mixture incubated 60 min at 22°C. The suspension was then layered on the oil and the radioactivity in the platelet pellet determined after centrifugation.

**Relative binding of IgG and  $F(ab)_2$  to platelets in the presence of malarial antigen.** 100  $\mu\text{g}$  of patient or control  $^{125}\text{I}$ -IgG or  $^{125}\text{I}$ - $F(ab)_2$  was diluted in 100  $\mu\text{l}$  of PBS containing 5% AB serum. This was incubated at 22°C for 30 min with either 100  $\mu\text{l}$  (100  $\mu\text{g}$ ) of unlabeled malarial antigen or a PBS control. 200  $\mu\text{l}$  of washed platelets ( $3.0 \times 10^7$ , final) were incubated with the serum and malarial antigen for 60 min at 22°C and layered on the oil as described. After centrifugation the platelet radioactivity was measured in a gamma counter.

## RESULTS

### *Patient studies*

28 patients (26 *P. vivax*, 2 *P. falciparum*) had simultaneous platelet counts and PAIgG determinations performed at presentation (all using the antiglobulin consumption assay). 17 were thrombocytopenic (platelet count < 150,000/ $\mu\text{l}$ ) and 16 of the 17 had elevated PAIgG. 5 of the 11 nonthrombocytopenic patients also had elevated levels of PAIgG. There was an inverse relationship between the platelet count and PAIgG level in the patients,  $r = 0.635$ ,  $P < 0.01$  (Fig. 1). 10 patients had serial platelet count and PAIgG determinations performed and in all cases the platelet count rose within 7 d of the start of treatment and the PAIgG level returned to normal as the platelet count rose.

Several of the thrombocytopenic episodes were severe, with platelet counts of <20,000/ $\mu\text{l}$ . These patients were given prophylactic platelet transfusions.

**Coagulation studies.** The APTT, prothrombin time, thrombin clotting time, and coagulation assays were performed in 11 patients and were normal in all except for one patient who had a prolonged APTT and a Factor XII level of 20%, and another who had an isolated increase in the level of FDP. The protamine sulphate paracoagulation assay was performed in eight patients and was normal in all. Four thrombocytopenic

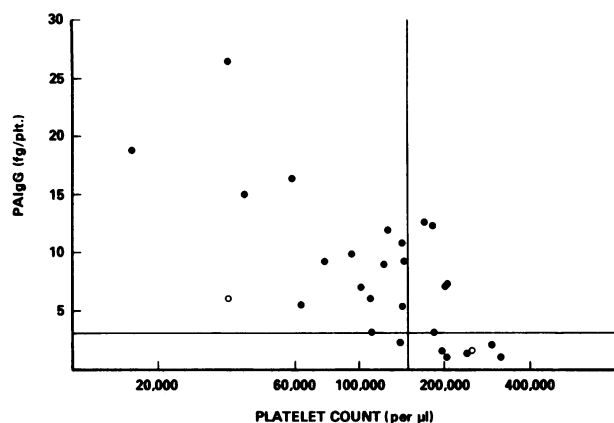


FIGURE 1 The relationship between the platelet count (abscissa) and the level of PAIgG measured using the antiglobulin consumption assay (ordinate) in 28 patients with *P. falciparum* (○) and *P. vivax* (●) infections. The intersecting lines indicate the limits of normal for the platelet count (vertical line) and PAIgG (horizontal line).

patients (nadir in platelet count of 16,000–25,000/ $\mu$ l) had serial fibrinopeptide A determinations performed during their hospitalization and all results were within the normal range for a hospitalized population (<3 pg/ml).

#### *In vitro studies*

##### *Malarial-dependent binding of IgG to platelets.*

The incubation of patient serum with 100  $\mu$ g of malarial antigen resulted in a significant increase in the binding of IgG to control platelets (Table I). This IgG was firmly bound and resisted multiple (three) washings. In contrast, control serum plus malarial antigen did not result in the increased binding of IgG to platelets, nor was there increased binding of patient serum to control platelets in the absence of malarial antigen. There was no pelletable precipitate formed by the incubation of malaria antigen and patient serum.

*Binding of malarial antigen to platelets.* Malarial antigen firmly bound to platelets in a dose-dependent fashion (Fig. 2). Experiments were performed using incubation times of 60, 120, and 180 min and all demonstrated that the reaction was complete by 60 min. If platelets were omitted, no antigen crossed the oil. The malarial antigen binding sites were saturable and incubation of 100  $\mu$ g of  $^3$ H-malaria antigen with a 100-fold excess of unlabeled antigen resulted in <5% binding of the labeled antigen to the platelets. Soluble radiolabeled erythrocyte antigen prepared from control radiolabeled erythrocytes not infected with malaria did not bind to the platelets.

*Effect of increasing concentrations of malarial antigen on the binding of  $^{125}$ I-IgG to platelets.* In-

TABLE I  
*Binding of IgG to Platelets using Patient or Control Serum from Healthy Individuals in the Presence or Absence of Malarial Antigens*

Serum	Malaria antigen (100 $\mu$ g)	Platelets (200,000/ $\mu$ l)	S-PBIgG fg IgG/platelet	n
Control	+	+	<3	8
Patient	+	–	–*	1
Patient	–	+	<3	8
Patient	+	+	5–15	8

+, indicates included in the experiment.

\*, no precipitate (immune complex) was noted.

n, number of experiments, each using four dilutions of platelets. Serum-platelet bindable IgG (S-PBIgG) was measured on three times washed platelets using the IRMA assay. The patient serum was a pool of several sera collected from patients with recurrent *P. falciparum* infections.

ing concentrations of malarial antigen up to 18  $\mu$ g resulted in increasing binding of  $^{125}$ I-patient IgG to platelets. Higher concentrations of malarial antigen were associated with less binding (Fig. 3). Less than 1% of  $^{125}$ I-IgG prepared from control serum bound to platelets at any concentration of malarial antigen.

*Relative binding of IgG and F(ab')<sub>2</sub> to platelets in the presence of malarial antigen.* The percent binding of patient  $^{125}$ I-IgG and  $^{125}$ I-F(ab')<sub>2</sub> in the presence

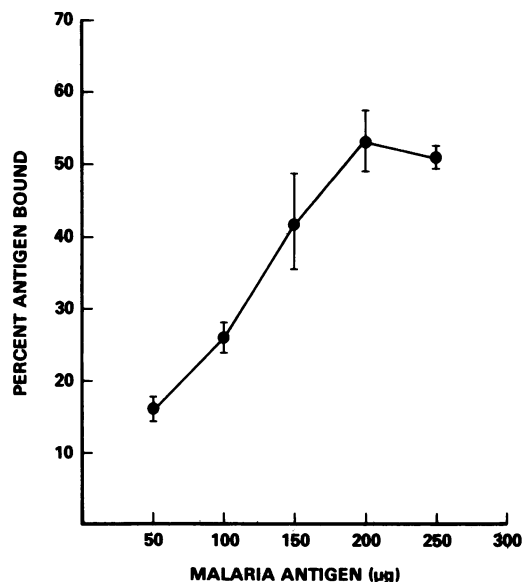


FIGURE 2 The binding of malarial antigen to washed human platelets. The percent antigen bound (determined from its specific activity) is displayed along the ordinate and the amount of antigen added is displayed along the abscissa. Each point represents the mean  $\pm$  SE of three or more experiments.

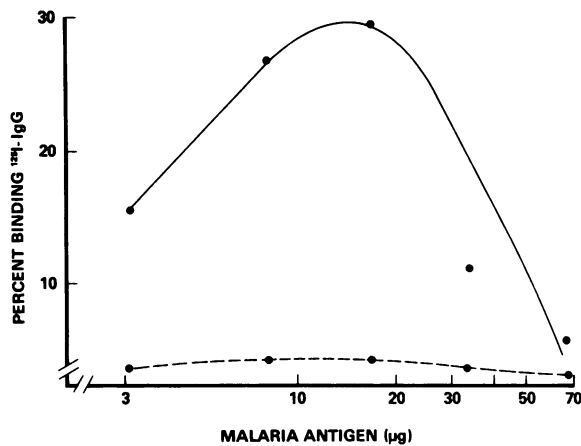


FIGURE 3 The relationship between the amount of patient (●—●) or control (●---●) <sup>125</sup>I-IgG (ordinate) that bound to a fixed number of platelets in the presence of increasing concentrations of unlabeled malarial antigen (abscissa).

of 100 µg of malarial antigen was 8.5 and 5.9%, respectively, or 0.5 and 0.4 nM, respectively. Less than 0.1% of either radiolabeled protein fraction bound to platelets in the absence of malaria. Similarly, <0.1% of control <sup>125</sup>I-IgG or <sup>125</sup>I-F(ab')<sub>2</sub> bound to platelets in the presence of malarial antigen.

### DISCUSSION

It has been recognized for many years that the majority of patients with malarial infections have thrombocytopenia, but the mechanism of the thrombocytopenia is unknown. Initially it was suggested that DIC was responsible, but more recently it has been shown that most patients with malaria do not have DIC (4-8). Consistent with these observations are the studies described in this report. Despite severe thrombocytopenia, most patients had no evidence of generalized thrombin action even when sensitive assays (fibrinopeptide A) were used. In contrast, the majority (16 of

SCHEMATIC REPRESENTATION OF HYPOTHETICAL WAYS IgG CAN BIND TO THE PLATELET IN MALARIA INFECTIONS	EXPERIMENTS USED TO CHALLENGE THE HYPOTHESIS	RESULTS OF THE EXPERIMENTS AND CONCLUSIONS CONCERNING THE HYPOTHESIS
<p>A. Non-specific binding of IgG following malaria-induced platelet damage.</p> <p>Altered Membrane</p>	<p>Measurement of the binding of IgG to platelets using patient serum or control serum with or without malaria antigen.</p>	<p>Increased binding of IgG to platelets required both patient serum and malaria antigen. The result is not consistent with this hypothesis.</p>
<p>B. Immune complex binding.</p> <p>Platelet Fc Receptor</p>	<ol style="list-style-type: none"> <li>The relative binding of intact IgG and F(ab')<sub>2</sub> fragments in presence of malaria antigen.</li> <li>Competitive displacement of antibody binding by increasing concentrations of malaria antigen.</li> </ol>	<ol style="list-style-type: none"> <li>Similar binding of F(ab')<sub>2</sub> and intact IgG to platelets. The result is not consistent with immune complex binding.</li> <li>Increasing concentrations of malaria antigen displaced platelet bound IgG. The result is consistent with immune complex binding.</li> </ol>
<p>C. Recognition of platelet bound antigen.</p>	<p>As above for hypothesis B.</p> <div style="border: 1px solid black; padding: 5px; width: fit-content; margin: 10px auto;"> <p>Y Y IgG Immunoglobulins * Malaria Antigen</p> </div>	<p>Both observations consistent with this hypothesis.</p>

FIGURE 4 Hypothetical mechanisms that could account for the elevated levels of PAIgG in patients with malarial infections.

17) of the thrombocytopenic patients had elevated levels of PAIgG during the thrombocytopenic episode. The PAIgG returned to normal as the thrombocytopenia resolved, and while the patient continued on the same antimalaria drugs, indicating that the thrombocytopenia was not drug induced. Elevated levels of PAIgG were also noted in 5 of 11 nonthrombocytopenic patients and these patients may have been in a compensated thrombocytolytic state (Fig. 1). There are several mechanisms that could account for the elevated PAIgG observed in patients with malaria and these are illustrated diagrammatically in Fig. 4. The possibility of nonspecific uptake of IgG by platelets damaged by malarial antigens was excluded by the demonstration that incubation of platelets and malarial antigen with control serum did not result in the binding of IgG to these platelets (Table I). Increased binding of IgG to control platelets occurred only when convalescent patient serum was incubated with malarial antigen and platelets.

The increased PAIgG could represent immune complexes adsorbed to platelet Fc receptors. Consistent with this hypothesis is the observation that patients with malarial infection frequently have circulating immune complexes (1). Alternatively, immune IgG could bind to platelet-bound malarial antigen, (Fig. 4). Indirect support for this latter hypothesis was provided by the demonstration that platelets have saturable binding sites for malarial antigen (Fig. 2). Increasing concentrations of malarial antigen were able to displace platelet-bound patient IgG. It was not determined why the IgG was displaced by concentrations of malarial antigen below that needed to saturate all platelet binding sites. The latter observation is consistent with either immune complex binding or immunoglobulin binding to platelet-bound malarial antigen.

To resolve this issue the malaria-dependent binding of intact patient IgG was related to the binding of the same IgG after enzymatic destruction of the Fc terminus to produce F(ab')<sub>2</sub>. These molecules would be unable to bind to the platelet Fc receptors and hence could be used to assay the relative contribution of immune complexes to the platelet-bound immunoglobulin. We found that the relative binding of patient IgG and F(ab')<sub>2</sub> was very similar and dependent upon the presence of malarial antigen.

In summary, in at least some patients with malaria, the thrombocytopenia is caused by immune mechanisms. Immune complexes are not responsible for the

elevated platelet-bound immunoglobulin, rather, IgG binds to platelet-associated malarial antigen through its Fab terminus.

#### ACKNOWLEDGMENTS

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