

NONIMMUNE I_gM, BUT NOT I_gG BINDS TO THE SURFACE OF *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTES AND CORRELATES WITH ROSETTING AND SEVERE MALARIA

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Abstract. Recent work suggests that IgG and IgM from nonimmune human serum (natural antibodies) bind to the surface of *Plasmodium falciparum*-infected erythrocytes and contribute to rosette formation by stabilizing the interaction between infected and uninfected erythrocytes. Here we show, in both laboratory clones and field isolates, that only IgM but not IgG is detected on the surface of infected cells. In field isolates, there was a strong positive correlation between IgM binding and rosette formation (Spearman's rank correlation coefficient $\rho = 0.804$, $P < 0.001$). Both rosette formation and IgM binding were associated with severe malaria, although statistical analysis indicates that rosette formation is the more strongly associated variable. Rosette formation, but not IgM binding, was also associated with malarial anemia. We conclude that IgM is the predominant class of natural antibodies binding to the surface of infected erythrocytes. However, we could not confirm previous suggestions that infected erythrocytes are coated with nonimmune IgG, which could lead to their interaction with host Fc γ receptors.

INTRODUCTION

Adhesion of erythrocytes infected with *Plasmodium falciparum* to endothelium (cytoadherence) and to uninfected erythrocytes (rosette formation) are thought to be important processes in the pathogenesis of severe malaria.¹ Only a minority of *P. falciparum* isolates show high levels of rosette formation, and it has been shown that rosette formation occurs more commonly in parasite isolates from patients with severe malaria than in those from patients with mild disease.^{2–4} Parasites that form rosettes cause greater obstruction to microvascular blood flow than parasites that do not form rosettes,⁵ and in this way may contribute to the disease process. Alternatively, rosette formation may enhance parasite growth and survival and therefore lead to higher parasitemias *in vivo* with an increased likelihood of severe disease.⁶ Therefore, rosette formation is a parasite virulence factor and could be a target for anti-disease therapies used to treat severe malaria.

Recent work has begun to identify the parasite ligands and uninfected red blood cell receptors that interact to bring about formation of rosettes. The variant *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) encoded by specific *var* genes is the parasite ligand for rosette formation^{7,8} and may interact with a number of different red blood cell receptors such as complement receptor 1 (CR1),^{7,9} heparan sulfate-like molecules,⁸ ABO blood group sugars,¹⁰ and CD36.¹¹ The occurrence of fibrillar structures connecting infected and uninfected erythrocytes in rosettes has also been described, and it was shown that the fibrillar structures were composed of at least partly of immunoglobulins from nonimmune human serum.¹² Immuno-electron microscopy and immunofluorescent labeling showed that both IgG and IgM bind to the surface of infected erythrocytes that formed rosettes, but not to uninfected erythrocytes that did not form rosettes.¹² Binding of IgG and IgM from normal human serum to infected erythrocytes that formed rosettes was also reported by other investigators.¹³ Most of the work to date has concentrated on two culture-adapted rosette-forming parasite clones/lines (PAR+ and TM284), although binding of IgG and IgM to field isolates has also been described.¹⁴

The aim of this study was to clarify the relationship be-

tween rosette formation and immunoglobulin binding by studying a wide range of *P. falciparum* culture-adapted parasites that differed in adhesion phenotype. Parasite clones/lines with one or more adhesion phenotype, including rosette formation, cytoadherence to endothelial cells, and platelet-mediated clumping of infected erythrocytes (autoagglutination)^{15,16} were studied using monoclonal antibodies in immunofluorescence assays (IFAs) to detect the presence of human immunoglobulins. In addition, *P. falciparum* field isolates were examined to determine the relationship between rosette formation, immunoglobulin binding, and disease severity.

MATERIALS AND METHODS

Parasites and culture. Laboratory lines (adapted to *in vitro* culture) TM180 and TM284 and clones (derived from a single cell) IT/R29 and PAR+ are as previously described.^{7,17} PAR+ has also been referred to as R+PA1 and FCR3S1 in some publications. Clones 3D7, 7G8, and T9/96 were obtained from Professor David Walliker (University of Edinburgh), and clones IT/A4, IT/C10, IT/A1R, and IT/A4R¹⁸ were obtained from Professor Chris Newbold and Dr. David Roberts (University of Oxford, Oxford, United Kingdom). Clone TM284S2¹⁹ was obtained from Professor Mats Wahlgren (Karolinska Institute, Stockholm, Sweden). Parasites were grown in group O erythrocytes by standard methods using RPMI 1640 medium supplemented with 25 mM glucose, 25 mM HEPES, 2 mM glutamine, 25 μ g/mL of gentamicin, and 10% pooled human serum, with the pH adjusted to between 7.2 and 7.4 with 1 M NaOH. Cultures were gassed with a mixture of 94% nitrogen, 5% carbon dioxide, and 1% oxygen. Field isolates were collected from children with a parasitemia of 0.3% or higher at Kilifi District Hospital (Kilifi, Kenya). Blood samples (1 mL) were collected into tubes containing EDTA. The cells were washed with RPMI 1640 medium three times and the buffy coat was removed. The isolates were then cultured as described in this report except that 10% human AB serum was used and the cultures were grown for at least 16 hours *in vitro* until at least 50% of the parasites were mature pigmented trophozoites or schizonts before use in experiments. Parasite maturity was assessed by Giemsa-

stained thin blood smears. All laboratory clones/lines were grown in a single pool of serum from six donors of assorted ABO types, and all field isolates were grown in a single pool of AB serum from two donors.

Patients. Field isolates were collected from severe and uncomplicated malaria patients after obtaining informed consent from the patient's family. All protocols involving human subjects were approved by the Kenya Medical Research Institute ethical review board. Severe malaria was defined by the presence of cerebral malaria (in coma and unable to localize a painful stimulus), respiratory distress (abnormally deep breathing), or prostration (unable to sit or, in infants, unable to breast feed).²⁰ These children were admitted to the high dependency unit for parenteral therapy. The uncomplicated malaria patients were children with an acute febrile illness and no features of severe malaria who were treated as outpatients, or inpatient children admitted to the pediatric ward, but not fulfilling the criteria for admission to the high dependency unit (i.e., no disturbance of consciousness, not prostrated, and not having respiratory distress). All of these children made an uneventful recovery on oral therapy.

Immunofluorescence assay to detect human immunoglobulins. The IFAs were carried out on parasite cultures at the mature pigmented trophozoite or schizont stage. Aliquots (50 μ L) of unfixed cells at a hematocrit of 2% were washed once in phosphate-buffered saline (PBS) and resuspended in PBS/1% immunoglobulin free bovine serum albumin (BSA) (Sigma, Poole, Dorset, United Kingdom). Mouse monoclonal antibodies (MAbs) to human IgG (Fc), IgG (Fab), IgM, IgA, IgD, IgE, IgG1, IgG2, IgG3, and IgG4 (Serotec, Kidlington, Oxon, United Kingdom) and matched isotype controls were added to give a final concentration of 1 μ g/mL and incubated for one hour on ice. Experiments with field isolates were carried out at room temperature using MAbs to IgG (Fc), IgM, and isotype controls only. Cells were gently resuspended every 10 minutes throughout the incubation. The cells were then washed twice with 750 μ L of PBS and resuspended in 50 μ L of a 1:200 dilution of Alexa Fluor™ 488 labeled goat anti-mouse IgG (highly purified; Molecular Probes, Leiden, The Netherlands) plus 1 μ g/mL of 4,6-diamidino-2-phenylindole (DAPI) to stain the parasite nuclei. For the image shown in Figure 1, 10 μ g/mL of concanavalin A-tetremethylrhodamine isothiocyanate (TRITC; Sigma, St. Louis, MO) was also added to stain the uninfected erythrocytes. Alexa Fluor™ 488 is equivalent to fluorescein isothiocyanate (FITC) but provides increased sensitivity and is more resistant to photo-bleaching. The cells were incubated for 45 minutes on ice (laboratory clones/lines) or at room temperature (field isolates) and were gently resuspended every 10 minutes. Cells were then washed twice with PBS as described in this report, resuspended in PBS/1% BSA at a hematocrit of 2%, and a wet preparation was viewed by fluorescence microscopy. Alternatively, after the cells were washed, they were resuspended at a hematocrit of approximately 30% in PBS/1% BSA and used to make a thin smear on a clean microscope slide. After air drying, the smear was overlaid with a drop of 1.25 mg/mL of diazabicyclo-[2,2,2]octane in 50% glycerol/50% PBS and a 22 \times 22 mm coverslip, and the edges of the coverslip were sealed with nail varnish. Initial experiments showed that cells prepared in this way gave the same results as wet preparations. All slides were read immediately; however, we subsequently showed that the thin

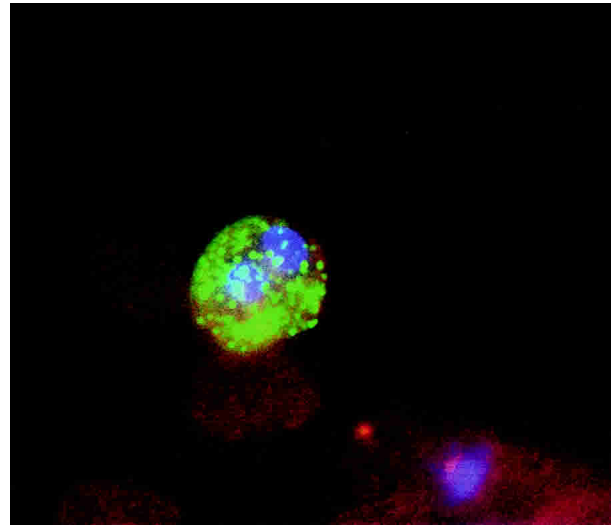


FIGURE 1. Immunofluorescence assay of *Plasmodium falciparum* line TM284 with a mouse monoclonal antibody to human IgM and Alexa Fluor™ 488 conjugated goat anti-mouse secondary antibody (green). Parasite nuclei are stained blue (4,6-diamidino-2-phenylindole) and erythrocytes are stained red (concanavalin A-tetremethylrhodamine isothiocyanate).

smears could be stored at 4°C for at least three months without losing their fluorescent signal. Therefore, this method is particularly useful for the study of field isolates in areas where facilities for fluorescence microscopy are not readily available. Experiments on laboratory clones/lines were carried out at least twice, with similar results being seen in repeated experiments. Slides were viewed on an Olympus BX-50 microscope (Southall, Middlesex, UK) and images were recorded using a digital camera and Openlab software (Improvision, Coventry, United Kingdom). The percentage of infected erythrocytes binding IgM was determined by counting at least 200 infected erythrocytes, and was carried out without knowledge of the rosette frequency or the clinical category of the sample. Additional experiments were carried out to look for IgG binding including direct immunofluorescence with a FITC-conjugated, affinity-purified rabbit anti-human IgG polyclonal reagent (F0056, 1:20 dilution; Dako, Ely, United Kingdom) and a FITC-conjugated rabbit immunoglobulin control (X0929, 1:20 dilution; Dako), and an indirect IFA as described in this report using a pool of MAbs to human IgG1, IgG2, IgG3, and IgG4 (all at a concentration of 1 μ g/mL) and the anti-IgG (Fc) and anti-IgG (Fab) MAbs at a concentration of 10 μ g/mL. The anti-human IgG (Fc) MAb used here is specific for the CH2 domain of human IgG, and the anti-human IgG (Fab) is specific for the CH1 domain. The rabbit anti-human IgG polyclonal reagent is specific for human IgG gamma chains.

Assessment of rosette formation. The frequency of rosette formation was assessed when the parasites had reached the pigmented trophozoite or schizont stage. A sample of parasite culture at a hematocrit of 1–2% was stained with ethidium bromide (25 μ g/mL) and a wet preparation was viewed with a fluorescence microscope using ultraviolet and visible light to visualize both stained and unstained cells simultaneously. Two hundred mature infected cells were counted, with the binding of two or more uninfected erythrocytes constituting a rosette. The frequency of rosette formation is the percentage

of mature infected cells that form rosettes. The clinical category of all samples was blinded until the end of the study.

Statistical analysis. Statistical analysis was carried out using StatView software (Abacus Concepts, Inc., Berkeley, CA). Data were analyzed with the Student's *t*-test, Spearman's rank correlation coefficient, and the Mann-Whitney U test as indicated in this report. Additionally, data were transformed and analyzed by multiple regression to investigate the relative contributions of rosette formation and IgM binding to severe malaria and anemia.

RESULTS

Human immunoglobulin binding by *P. falciparum* laboratory clones/lines. To assess immunoglobulin binding and to determine whether it is associated only with rosette formation as previously reported,^{13,14,21} or occurs in other cytoadherent or autoagglutinating parasites, we studied 12 parasite clones/lines of differing adhesion phenotype (Table 1). Fresh and mock-cultured uninfected erythrocytes were also studied as controls. Immunoglobulin binding to the surface of live infected cells was detected by IFA using MAbs to human IgG (Fc), IgG (Fab), IgM, IgA, IgD, and IgE and relevant isotype controls. We observed IgM binding to the surface of infected erythrocytes in three of five parasite clones/lines with a high level of rosette formation, and in three clones/lines with a low frequency of rosette formation (Table 1 and Figure 1). A bright punctate pattern over the surface of infected cells was seen (Figure 1), consistent with the localization of IgM at the knobs on the infected erythrocyte surface where PfEMP1 is found.^{22,23} The percentage of IgM-positive infected erythrocytes and the percentage of rosette-forming infected erythrocytes were similar in each parasite clone/line (Table 1), and the IgM-positive infected erythrocytes in wet preparations were seen to be in rosettes. Although rosette formation is associated with IgM binding in some parasites, but it is apparent that rosette formation can also occur in the absence of IgM (see parasite clones/lines IT/R29 and TM180 in Table 1).

TABLE 1
Rosette formation and IgM binding in *Plasmodium falciparum* laboratory clones/lines

Parasite clone/line	Adhesion phenotype*	Rosette frequency† (%)	IgM binding‡ (%)
PAR+	C+ R+ A-	46	44
TM284	C+ R+ A-	38	30
TM284S2‡	C+ R+ A-	53	51
TM180	C+ R+ A-	49	0
IT/R29	C+ R+ A-	60	0
IT/A1R	C+ R+ A-	41	42
IT/A4R	C+ R+/- A-	18	15
IT/A4	C+ R+/- A-	3	4
IT/C10	C+ R+/- A+	2	3
3D7	C+ R- A-	0	0
7G8	C+ R- A-	0	0
T9/96	C- R- A-	0	0
Fresh RBC control	NA	NA	0
Mock-cultured RBC control	NA	NA	0

* C = cytoadherence; + binds to at least one endothelial receptor; R = rosette forming; +, >20% of infected erythrocytes in rosettes; +/-, 1-19% of infected erythrocytes in rosettes; A = autoagglutination (binding of infected erythrocytes to other infected erythrocytes); +, >20% of infected erythrocytes in agglutinates; RBC = red blood cell; NA = not applicable.

† Mean of at least two experiments.

‡ TM284S2 is a clone derived from TM284.¹⁹

In the autoagglutinating clone IT/C10, IgM-positive cells were always seen in rosettes and not in autoagglutinates, indicating that IgM binding does not play a part in the phenomenon of platelet-mediated clumping of infected erythrocytes.¹⁶

When tested with MAb reagents, we did not detect specific binding of IgG to infected erythrocytes in any of the parasite cultures examined, including the clones/lines TM284, TM180, IT/R29, and TM284S2 that have been reported previously to bind IgG.^{12,19,21} We did observe very low levels of IgG, IgM, and IgA on both infected and uninfected erythrocytes in all parasite cultures and erythrocyte controls. This was seen as a faint scattering of fine dots over the cell surface (0-10 dots per cell, with 20-50% of cells showing at least one dot) that was readily visible, but difficult to reproduce photographically. Such fine dots were not seen with the isotype control MAbs or with MAbs to IgD or IgE. When tested with MAbs to IgG subclasses, faint dots similar to those seen with the IgG (Fc) and IgG (Fab) MAbs were seen with IgG1, IgG2, and IgG3, but not IgG4. These faint positive signals with antibodies to IgG, IgA, and IgM represent the low levels of immunoglobulins present on uninfected red blood cells.²⁴ Our ability to detect these low levels of IgG on uninfected erythrocytes indicates that our assay is sensitive and we should therefore be able to detect IgG bound to infected erythrocytes if it is present. To exclude the possibility that our anti-human IgG MAb reagents were of restricted specificity and we were therefore failing to detect bound IgG, we also tested a FITC-conjugated affinity-purified rabbit polyclonal anti-human IgG reagent obtained from Dako. When tested with the polyclonal reagent in a direct IFA, parasite clones/lines TM284, TM180, IT/R29, and TM284S2, which have been previously reported as binding IgG,^{12,19,21} remained negative for IgG in all our experiments. We also tested the anti-human IgG MAbs in a pool and at higher concentrations (10 µg/mL), and again failed to detect any evidence for IgG binding specifically to infected erythrocytes. We therefore found no evidence to support a role for natural IgG antibodies in rosette formation, platelet-mediated autoagglutination, or cytoadherence.

Human immunoglobulin binding by *P. falciparum* field isolates. We also studied IgM and IgG binding in *P. falciparum* field isolates from patients with severe and uncomplicated malaria in Kilifi, Kenya (a summary of patient information is shown in Table 2). Out of 82 field isolates collected, 79 grew to maturity. Three isolates were excluded from the study due to bacterial contamination, one isolate was excluded because widespread clumping of uninfected red blood cells made it impossible to assess rosette formation, and one isolate was excluded due to extensive lysis of infected erythrocytes. Rosette formation was assessed in 74 isolates and IgG and IgM binding was assessed in 56 isolates. The remaining 18 isolates were not examined for immunoglobulin binding due to time constraints. These 18 isolates were mainly from uncomplicated malaria cases (16 of 18) and all had frequencies of rosette formation of less than 15%.

As seen with the laboratory clones/lines, we did not detect IgG binding specifically to infected erythrocytes in any of the field isolates studied. However, IgM binding was detected in many isolates, and there was a strong positive correlation between the percentage of infected erythrocytes binding IgM and the percentage of infected erythrocytes in rosettes

TABLE 2
Details of patients from whom *Plasmodium falciparum* field isolates were obtained

Disease category	Number of cases	Mean age, months (range)	Mean hemoglobin level (g/dL) (range)	Mean parasitemia (%) (range)
Severe	25	27.1* (3–75)	6.6† (2.8–12.0)	12.1‡ (0.6–36.0)
Uncomplicated	49	32.5* (8–85)	8.3† (2.7–12.0)	7.2‡ (0.3–42.0)

* $P = 0.33$, by Student's *t*-test.

† $P = 0.006$, by Student's *t*-test.

‡ $P = 0.02$, by Student's *t*-test.

(Figure 2, Spearman's rank correlation coefficient $\rho = 0.804$, $P < 0.001$). Exclusion of the outlying value did not alter the statistical significance of the correlation ($\rho = 0.793$, $P < 0.001$). All isolates that formed rosettes also bound IgM, whereas a small proportion of isolates (7 of 56) did not form rosettes but showed low levels of IgM binding (2–12%, Figure 2).

Rosette formation and IgM binding and disease severity.

As described previously in this population,⁴ higher levels of rosette formation were seen in isolates from patients with severe malaria than in isolates from uncomplicated malaria controls, although there was considerable overlap between the two samples (Figure 3A; severe isolates, $n = 25$, median frequency of rosette formation = 14%, interquartile range = 4–32%; uncomplicated isolates, $n = 49$, median frequency of rosette formation = 3.5%, interquartile range = 1–10%, $P < 0.001$, by Mann-Whitney U test). There was also a trend towards more rosettes in the inpatient uncomplicated cases ($n = 30$, median frequency of rosette formation = 4%, interquartile range = 1–10%) compared with the outpatient uncomplicated cases ($n = 19$, median frequency of rosette formation = 1%, interquartile range = 0–7%) as previously observed;⁴ however, this was not statistically significant ($P = 0.1$, by Mann-Whitney U test). The data on rosette formation collected in the current study in the year 2000, and in a previous study in the same population in 1993⁴ are remarkably similar, indicating that the incidence of rosette formation and its association with severe disease are extremely stable over time.

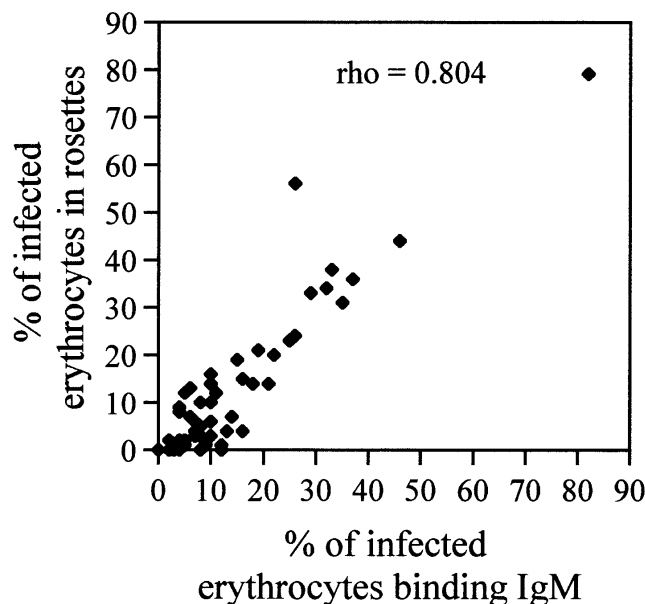


FIGURE 2. Scatter diagram showing the relationship between rosette formation and IgM binding in 56 *Plasmodium falciparum* field isolates. Each point represents one isolate.

In view of the strong correlation between rosette formation and IgM binding, it was not surprising to find that IgM binding is also significantly associated with severe malaria (Figure 3B; severe isolates, $n = 23$, median percentage of infected erythrocytes binding IgM = 15%, interquartile range = 7–31%; uncomplicated isolates, $n = 33$, median = 8%, in-

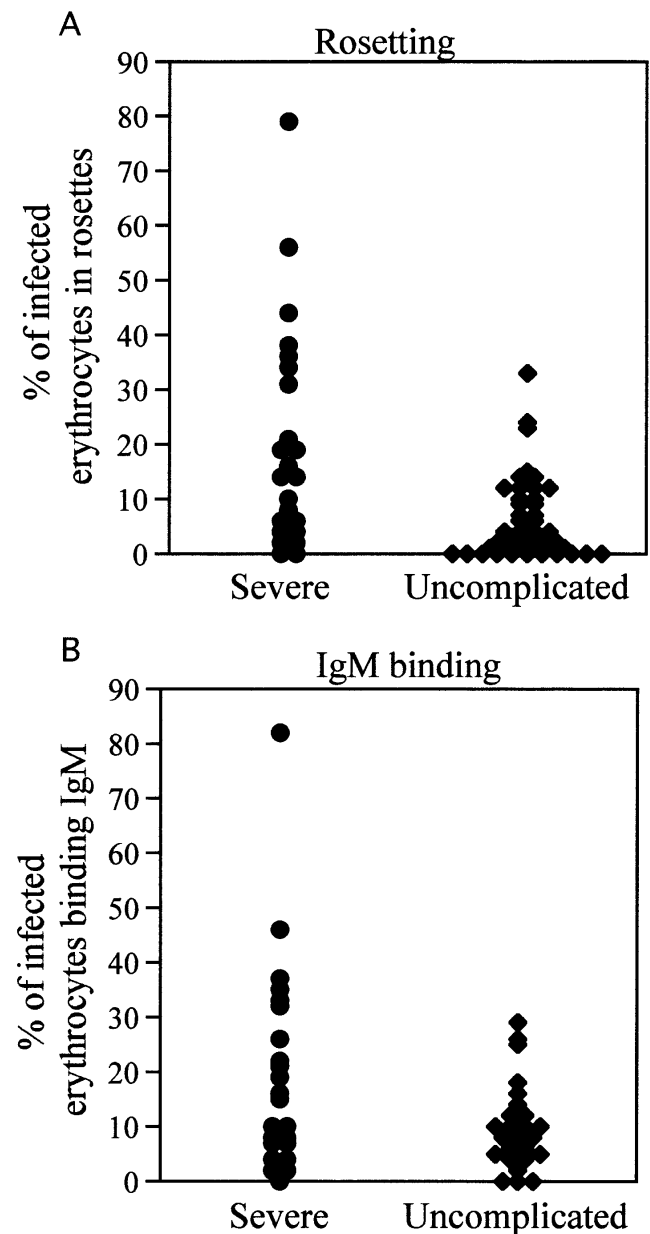


FIGURE 3. Rosette formation (A) and IgM binding (B) in *Plasmodium falciparum* field isolates from severe and uncomplicated malaria patients. Each point represents one isolate.

terquartile range 5–12%, $P < 0.05$, by Mann-Whitney U test). This significant association between IgM binding and disease severity was seen despite the fact that many uncomplicated malaria isolates with low frequencies of rosette formation (and by implication, low IgM binding) were not assessed for IgM binding due to time constraints as described earlier. To determine the contribution of rosette formation to disease severity independent of IgM, we normalized the data by square root transformation, and carried out an analysis of variance with a covariate for IgM included in the model. When this was done, rosette formation remained significantly higher in severe disease isolates than in uncomplicated disease isolates ($P = 0.011$). However, when IgM binding was analyzed after standardizing for rosette formation, IgM binding was no longer significantly associated with severe disease ($P = 0.387$).

Rosette formation and IgM binding and anemia. Previous work suggested that a subgroup of Gabonese children with moderate anemia (hemoglobin (Hb) level between 5 and 9 g/dL) had high levels of immunoglobulin on the surface of infected erythrocytes, although statistically significant differences between anemic and non-anemic children were not observed.¹⁴ We therefore studied the relationship between rosette formation, IgM binding and Hb level in this sample of parasite isolates from Kenyan children to determine if there was any evidence for an association between IgM binding and malarial anemia. It should be noted that in this Kenyan population, anemia (Hb < 5 g/dL) is not on its own a factor indicating severe malaria because in the absence of other factors indicating severe disease such as impaired consciousness, respiratory distress, and prostration, having a Hb level less than 5 g/dL is not indicative of a poor prognosis.²⁰ We found a weak negative correlation between both rosette formation and Hb (Figure 4A; Spearman's rank correlation coefficient $\rho = -0.297$, $P = 0.013$) and IgM and Hb (Figure 4B; $\rho = -0.272$, $P = 0.05$). However, after exclusion of the outlying value, only rosette formation remained significantly correlated with the Hb level, (rosette formation, $\rho = -0.273$, $P = 0.024$; IgM binding, $\rho = -0.242$, $P = 0.084$). When comparisons were made of anemic (Hb < 5 g/dL) versus non-anemic (Hb > 5 g/dL) children, rosette formation, but not IgM binding, was significantly higher in the anemic children (rosette formation in anemic isolates, $n = 14$, median frequency of rosette formation = 19%, interquartile range = 8–36%; non-anemic isolates, $n = 57$, median = 4%, interquartile range = 1–12%, $P < 0.001$, by Mann-Whitney U test; IgM binding in anemic isolates, $n = 12$, median IgM binding = 17%, interquartile range = 7–32%; non-anemic isolates $n = 41$, median = 9%, interquartile range = 5–14%, $P = 0.061$, by Mann-Whitney U test). Finally, multiple regression analysis was carried out after square root transformation of the rosette formation and IgM binding data. It was found that rosette formation ($P = 0.001$), but not IgM binding ($P = 0.245$), was significantly associated with the Hb level after standardizing for the other variable.

DISCUSSION

The main finding of this study is that IgM, but not IgG, from nonimmune serum binds to infected erythrocytes in *P. falciparum* cultures, and that there is strong positive correla-

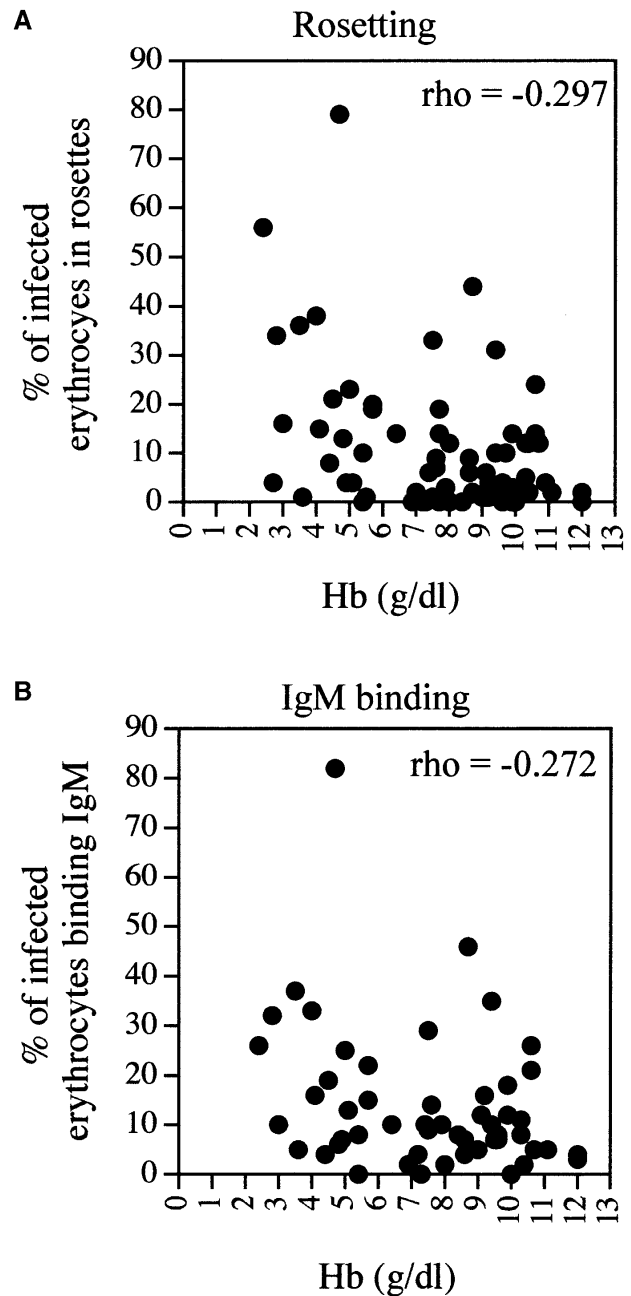


FIGURE 4. Scatter diagrams showing the relationship between rosette formation and hemoglobin (Hb) level (A) and IgM binding and Hb level (B) in *Plasmodium falciparum* field isolates. Each point represents one isolate.

tion between IgM binding and rosette formation in field isolates. Although two laboratory clones/lines were shown to form rosettes without binding IgM, all of the field isolates studied that formed rosettes also bound IgM, suggesting that the two phenomena are closely linked. A subgroup of field isolates (7 of 56) bound IgM without rosette formation, indicating that IgM binding alone is not sufficient to cause rosette formation.

Previous studies have shown both IgG and IgM natural antibodies binding to infected erythrocytes that form rosettes.^{12–14,21} We have studied an extensive range of laboratory clones/lines (including many previously reported as IgG

positive) and field isolates using both MAbs and affinity-purified polyclonal antibodies to human IgG. Although we detected low levels of IgG binding to uninfected erythrocytes, we did not detect any evidence for specific binding of IgG to infected erythrocytes. Several possible explanations can be put forward for our inability to corroborate the findings of previous studies. First, it is possible that the IgG natural antibodies that were shown to bind to infected erythrocytes in other studies are not present in all sera. We cannot exclude this possibility; however, we have repeated the IFA experiments using the parasite line TM284 grown in five different serum pools, each made up of serum from at least five individuals, and obtained consistent results in all cases i.e., positive for IgM, negative for IgG. Second, it is possible that the use of heat-inactivated serum may have given artifactual results in previous studies. We have found increased amounts of IgG when parasite cultures are grown in heat-inactivated serum; however, the IgG is seen on both uninfected and infected erythrocytes. Finally, the polyclonal antibodies used to detect human IgG in previous studies^{12–14,21} may have given false-positive results, particularly when used at high concentrations. We have found that uninfected sera from some (but not all) rabbits, sheep, and goats show surface fluorescence of infected erythrocytes in the IFA with some *P. falciparum* clones and field isolates. This cross-reactivity is a property of whole sera or immunoglobulins purified from whole sera, and is not observed with a variety of affinity-purified rabbit polyclonal antibodies (Rowe JA, unpublished data). Other studies have also reported cross-reactions between some rabbit sera and the surface of infected erythrocytes.^{25,26} Thus, the results of the IFA with *P. falciparum*-infected erythrocytes can be influenced by the antibodies used and their degree of purity. We suggest that unequivocal results can only be obtained using MAbs or affinity-purified polyclonal antibodies, accompanied by appropriate controls to exclude false positives.

A recent paper has suggested that nonimmune IgG binding to the surface of infected erythrocytes is also important in placental infection during malaria in pregnancy.¹⁹ We did not examine any placental parasites during the current study, whose primary aim was to clarify the role of natural antibodies in rosette formation. However, we did examine the parasite clone TM284S2 (a clone that forms rosettes) that was used by Flick and others¹⁹ to demonstrate placental binding and IgG binding. Once again, we detected IgM, but not IgG. Due to the absence of IgG, we believe that it is unlikely that infected erythrocytes interact with host Fc γ receptors as suggested by Flick and others,¹⁹ but it is still possible that interaction with Fc μ receptors could occur. The distinction shown here between IgG and IgM is not merely academic because if it does lead to interaction with host Fc receptors, Fc γ receptors are more widely distributed and may have different physiologic effects compared with Fc μ receptors.

The strong positive correlation between rosette formation and IgM binding shown in this study does not prove a causal relationship between the two phenomena. However, recent work does suggest that the binding of IgM has important consequences in terms of stabilizing the interactions between cells in a rosette. With clone PAR+, rosette formation does not occur in the absence of IgM, and rosettes form when purified IgM is added back to IgM-deficient medium.^{13,14} Also, the purification of subcomponents of serum showed that IgM plays an important role in rosette formation of clone

PAR+.²⁷ Antibodies to immunoglobulins have been shown to inhibit rosette formation in field isolates from Kenya,⁹ and many field isolates do not form rosettes in the absence of human serum.²⁸ A recent study has also shown that high serum concentrations strengthen binding forces in rosettes and enable them to withstand shear forces equivalent to those seen in post-capillary venules in the human circulation *in vivo*.²⁹ The identity of the serum components responsible for enhancement of rosette formation was not examined. However, it seems plausible that the serum-strengthening effect could be mediated by IgM, although other serum proteins with red blood cell-aggregating properties may also play a role.²¹

The exact nature of the ligand(s) for IgM on infected erythrocytes is currently under investigation. A recent study indicates that IgM may bind to one variant of the PfEMP1 family, and demonstrates that part of the semi-conserved head structure of PfEMP1 (the cysteine-rich interdomain region, CIDR) mediates the binding of IgM in the parasite clone FCR3S1.2.³⁰ It remains to be confirmed whether this interaction with the CIDR of PfEMP1 occurs in other IgM binding in parasites that form rosettes, although recent data suggest that a different domain of PfEMP1 (the DBL2 β domain) binds IgM in the parasite clone TM284S2.¹⁹ If IgM molecules are stabilizing rosettes, it is apparent that IgM must be interacting with receptors on the uninfected erythrocytes, as well as with a specific ligand on infected cells. Low titers of natural antibodies are observed in nonimmune human serum that recognize red cell blood surface molecules, such as the blood group I antigen,³¹ autologous ABO antigens,³² and phospholipids.³³ However, the precise specificity of natural IgM antibodies that bind to infected erythrocytes has not yet been determined, and the molecules that they recognize on uninfected erythrocytes are unknown.

The immunologic consequences of IgM binding to the surface of infected erythrocytes also remains to be clarified. A poly-immunoglobulin receptor that binds the J chain of IgM and IgA is present on B lymphocytes,³⁴ and an Fc μ receptor that may function in antigen presentation and phagocytosis and has recently been described on B lymphocytes and macrophages.³⁵ Whether opsonization of infected erythrocytes by IgM natural antibodies leads to interaction with B cells or macrophages is unknown, but it seems unlikely that the parasite would maintain a mechanism of binding that could contribute to its own destruction by the hosts' immune system. However, the situation could be analogous to the PfEMP1-mediated binding of infected erythrocytes to CD36 on dendritic cells.³⁶ This process interferes with dendritic cell activation and therefore represents a parasite mechanism for inducing host immunosuppression. Another possible immunologic consequence of IgM binding is complement activation that could lead to complement-mediated lysis of infected erythrocytes or phagocytosis via complement receptors. Interactions between natural antibodies, complement, and microorganisms are thought to be important in immune responses to a variety of hematogenically spread pathogens.³⁷ The possible involvement of complement in IgM binding and rosette formation is particularly intriguing, given the evidence that complement receptor 1 (CR1) is a rosette formation receptor on uninfected erythrocytes,⁷ and CR1 interacts with *P. falciparum*-infected cells via its complement binding sites.⁹ However, we have shown that complement is not required for

the formation of rosettes,⁹ and preliminary experiments show no evidence for complement activation on infected erythrocytes from either IgM-binding or non-IgM binding parasite lines (Rowe JA, unpublished data). Rather than being disadvantageous, it is also possible that IgM binding is beneficial to the parasite. For example, the presence of a coating of non-immune IgM could sterically interfere with the binding of specific antibodies to *P. falciparum* surface proteins that are thought to be crucial for host immunity and parasite clearance.^{38–41} Further investigations are required to determine the exact functional significance of the natural IgM antibodies that bind to infected erythrocytes that form rosettes.

The other major finding from this study is that IgM binding and rosette formation (as demonstrated previously^{2–4}) are associated with severe malaria. Statistical analysis indicates that rosette formation is the variable most strongly linked to severe disease, since IgM binding was no longer significantly associated with severity after standardizing for rosette formation. This suggests that rosette formation is more likely to be a causal factor in severe malaria than IgM binding, although causality cannot be proven by the current work. It seems plausible that IgM may contribute to malaria pathogenesis by stabilizing rosettes as discussed earlier. However, when IgM binding occurs in the absence of rosette formation, this is not associated with disease severity.

We have also shown that rosette formation is associated with anemia, a finding that has been reported previously in the Kenyan population.⁴² The precise mechanism underlying this association is unclear, but it could be that the uninfected erythrocytes in rosettes become damaged or altered in some way that leads to their clearance. Alternatively, it may be that rosette formation promotes high parasitemias *in vivo*,⁶ which subsequently leads to anemia.

The role of immunoglobulin in malarial anemia remains controversial and is the subject of extensive debate in the literature (e.g.,^{43,44}). Only one paper has suggested a role for immunoglobulins binding specifically to infected erythrocytes in the pathogenesis of malarial anemia.¹⁴ We found no statistically significant correlation between IgM binding and the Hb level after standardizing for rosette formation. Therefore, our data do not support the hypothesis of Scholander and others¹⁴ that specific binding of immunoglobulins to infected erythrocytes contributes to the development of malarial anemia.

In summary, we conclude that IgM is the predominant class of immunoglobulin binding to the surface of rosette-forming infected erythrocytes. Further work is needed to elucidate the exact specificity of the bound IgM and to determine if it is possible to disrupt the interaction between the infected cell and IgM and so destabilize interactions with host cells that may contribute to microvascular obstruction and severe malaria.

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