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ORIGINAL ARTICLE

In vitro RBC exposure to *Plasmodium falciparum* has no effect on RBC antigen expression

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SUMMARY. Severe malarial anaemia is a leading cause of death in African children younger than 3 years of age who are infected with *Plasmodium falciparum*. The pathogenesis of this anaemia is not understood. The purpose of this study was to determine if *P. falciparum* induces changes in RBC membranes that contribute to the immune destruction of RBCs. RBCs were collected from healthy subjects and tested using standard haemagglutination assays for 45 antigens representing 21 blood group systems/collections before and after exposure to *P. falciparum*, strain FVO. Lectins were used to determine whether crypt or neoantigens were expressed on the RBC membrane. Polybrene was used to detect changes in sialic acid. RBCs were cultured *in vitro* with and without the parasite, and blinded serologic studies were completed. CD35 (complement receptor 1), CD55 (decay-accelerating factor), CD59 (membrane inhibitor of reactive lysis) and CD47 (integrin-associated protein) flow cytometric assays were compared for infected and uninfected RBCs. The percentage of parasitaemia was determined using Giemsa-stained thin blood films. Two (Ch, Lu^b) of the 45 antigens had differing strengths of agglutination between infected and uninfected RBCs, but these differences were resolved with a second source of antisera. Forty-three antigens showed no significant differences in the strength of agglutination between the infected and uninfected RBCs. Lectin and polybrene testing showed no differences. CD35, CD55, CD59 and CD47 levels showed no significant differences. *P. falciparum* does not appear to alter the expression of classified immunogenic antigens on the RBC membrane in this *in vitro* system. The pathogenesis of the haemolytic episode that occurs in these children remains unclear.

Key words: erythrocyte antigens, malarial anaemia, *Plasmodium falciparum*.

Cerebral malaria and severe malarial anaemia owing to *Plasmodium falciparum* infection are a leading cause of mortality in sub-Saharan Africa. Severe *P. falciparum* anaemia most often affects children and pregnant women and is the leading cause of death in children younger than 3 years of age (Miller *et al*., 1994). The pathogenesis of malarial anaemia is not completely understood, but it is likely that several mechanisms are involved. Red blood cell (RBC) destruction during schizont rupture, suppression of erythropoiesis and destruction of uninfected RBCs have all been implicated in malarial anaemia (Facer *et al*., 1979; Abdalla *et al*., 1980; Abdalla & Weatherall, 1982; Merry *et al*., 1986; Weatherall *et al*., 1983; Weatherall, 1988; Abdalla, 1990; Kurtzhals *et al*., 1997).

In children with severe *P. falciparum* anaemia, haemoglobin levels fall to less than $5{\cdot}0\,\mathrm{mg\,dL^{-1}}$ despite low levels of parasitaemia (Abdalla *et al*., 1980; Weatherall *et al*., 1983; Weatherall, 1988; Abdalla, 1990). The fall in haemoglobin is too brisk to be owing to the suppression of erythropoiesis, and parasite levels are too low for the anaemia to be owing to the haemolysis of only infected RBCs, suggesting that uninfected RBCs are also destroyed. Uninfected RBCs are probably removed by immune mechanisms.

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Waitumbi and colleagues compared RBCs from Kenyan children with severe *P. falciparum* anaemia with RBCs from asymptomatic children. They found that RBCs from the children with severe *P. falciparum* anaemia had increased surface immunoglobulin G (IgG) and changes in the expression of complement regulatory receptors (Waitumbi *et al.*, 2000). Complement receptor 1 (CD35) and decay-accelerating factor (CD55) expression were decreased, and membrane inhibitor of reactive lysis (CD59) expression was increased (Waitumbi *et al.*, 2000). Goka and colleagues found increased RBC surface C3d levels in children from Ghana with severe *P. falciparum* anaemia (Goka *et al.*, 2001).

Although the immune response has been implicated in severe *P. falciparum* anaemia, the nature of the antibodies responsible for the anaemia is not known. During *P. falciparum* RBC invasion and *P. falciparum* replication, several membrane changes occur, including the deposition of *P. falciparum* proteins (Gratzer & Dluzewski, 1993). Antibodies causing anaemia may be directed to parts of the membrane of infected RBCs that have changed, or to *P. falciparum* proteins within the RBC membranes. However, in severe malarial anaemia, uninfected RBCs are also haemolysed. *P. falciparum* proteins are secreted by infected RBCs (Gratzer & Dluzewski, 1993), and it is possible that the antibodies could be directed to soluble *P. falciparum* proteins that adhere to uninfected red cells. Alternatively, proteins released by RBCs infected with *P. falciparum* may induce changes in uninfected RBCs that create neoantigens or crypt antigens that are the target of complement-fixing antibodies. The purpose of this study was to determine if *P. falciparum* infections induce changes in uninfected RBCs that affect antigen expression or create neoantigens or crypt antigens that contribute to the immune destruction of RBCs.

MATERIALS AND METHODS

Study design

RBCs were collected from healthy subjects and tested using standard haemagglutination assays for 45 antigens representing 21 blood group systems/collections before and after exposure to *P. falciparum*. Lectins were used to determine whether crypt or neoantigens were expressed on the RBC membrane. Polybrene was used to detect changes in sialic acid. RBCs were cultured *in vitro* with and without the parasite, and blinded serologic studies were performed when the cultures were complete. Flow cytometry was used to compare the expression on *P. falciparum*-exposed and unexposed RBCs of CD35, CD55, CD59 and CD47 (integrin-associated protein).

Materials

Reagent-grade antisera were obtained from BCA (West Chester, PA, USA), Gamma Biologicals, Inc. (Houston, TX, USA), Immucor, Inc. (Norcross, GA, USA) and Ortho Clinical Diagnostics, Inc. (Raritan, NJ, USA) (Table 1). Our reference laboratory provided the rare antisera except for anti-Wr^a which was provided by the American Red Cross. Mouse monoclonal anti-Wr^b was obtained from Sigma Chemical Company (St. Louis, MO, USA). Sheep antimouse IgG was obtained from Cappel (Durham, NC, USA). Anti-Fy3 was provided by Walter Reed Army Medical Center (Washington, DC, USA), and one source of anti-Ch was provided by Navy National Medical Center (Bethesda, MD, USA).

Reagent-grade RBCs were obtained from Gamma, Immucor and Ortho. Rabbit anti-IgG was obtained from Ortho. Hexadimethrine bromide used in the polybrene tests was obtained from Sigma. Fluorescein isothiocyanate (FITC)-conjugated mouse antihuman monoclonal antibodies were obtained from Pharmingen International (San Diego, CA, USA) and Caltag Laboratories (Burlingame, CA, USA).

Blood collection

After obtaining informed consent, whole blood was collected from three volunteers with group O, A, or B blood. Blood from the donor with group O RBCs was studied twice. The blood samples were collected in either acid citrate dextrose (ACD) or citrate phosphate dextrose (CPD) for haemagglutination tests performed before culture with *P. falciparum*. When RBCs were cultured with *P. falciparum*, whole blood was drawn directly into 50-mL syringes with CPD, and plasma was removed prior to culture.

P. falciparum culture

Whole blood was collected using a syringe with CPD (10%) as an anticoagulant. The blood was centrifuged and the plasma separated. RBCs were washed twice with RPMI 1640 medium without serum. After the second wash, an equal volume of RPMI complete medium was added to the RBC pellet to obtain a 50% RBC suspension. RPMI complete medium was prepared by adding to RPMI heat-inactivated human serum at a final concentration of 10%. The serum was from a donor with an ABO blood group compatible

Table 1. Comparison of red blood cell (RBC) antigen expression for red blood cells with and without exposure to *Plasmodium falciparum*

				Exposed cells				Naïve cells			
Antigen tested	ISBT	Reagent source	Reagent type	O1	O2	A_2	B	O1	O2	A_2	B
A	ABO1	Ortho	M	0	0	4	0	0	0	4	0
B	ABO2	Ortho	M	0	0	0	4	0	0	0	4
A, B	ABO3	Ortho	M	0	0	4	4	0	0	4	4
M	MNS1	Gamma	M	4	2	3	3	4	2	3	3
N	MNS2	Gamma	M	0	0	0	0	0	0	0	0
S	MNS3	Ortho	P	0	0	0	3	0	0	0	2
s	MNS4	Gamma	P	3	3	3	0	3	3	3	0
U	MNS5	In house	P	4	1	NT	NT	4	2	NT	NT
En^a	MNS28	In house	P	NT	1	NT	NT	NT	2	NT	NT
P1	P1	Immucor	M	0	0	1	1	0	0	1	1
D	RH1	Ortho	M/P	3	3	4	4	3	3	4	4
C	RH2	Ortho	M	4	4	4	4	4	4	4	4
E	RH3	Ortho	M	4	4	4	4	4	4	4	4
c	RH4	Ortho	M	4	4	4	4	4	4	4	4
e	RH5	Ortho	M	3	4	4	4	3	4	4	4
C^w	RH7	Gamma	S§	0	0	0	0	0	0	0	0
$Lu^b(1)$	LU2	In house	P	2	NT	NT	NT	0	NT	NT	NT
$Lu^b(2)$	LU2	In house	P	2	2	NT	NT	2	2	NT	NT
K	KEL1	Immucor	M	0	0	0	0	0	0	0	0
k	KEL2	Ortho	P	4	4	3	3	3	4	3	3
Kp^a	KEL3	BCA	P	0	0	0	0	0	0	0	0
Kp^b	KEL4	BCA	P	2	3	1	2	2	3	2	2
Le^a	LE1	Ortho	M	0	0	0	2	0	0	0	2
Le^b	LE2	Ortho	M	3	2	2	0	3	1	2	0
Fy^a	FY1	Immucor	P	0	0	0	0	0	0	0	0
Fy^b	FY2	Immucor	P	3	3	3	3	2	2	3	3
Fy3	FY3	WRAMC*	M	2	4	3	3	2	4	3	3
Fy6	FY6	In house	M	2	2	1	1	2	2	1	1
Jk^a	JK1	Immucor	P	0	0	3	3	0	0	3	3
Jk^b	JK2	Ortho	M	3	4	3	3	3	4	3	3
Di^b	DI2	In house	P	1	3	NT	NT	W	3	NT	NT
Wr^a	DI3	ARC	P	0	0	NT	NT	0	0	NT	NT
Wr^b	DI4	Sigma	M	3	3	3	3	3	3	3	3
Yt^a	YT1	In house	P	2	2	NT	NT	2	2	NT	NT
Co^a	CO1	In house	P	3	3	NT	NT	3	3	NT	NT
LW	LW	In house	P	2	3	NT	NT	2	3	NT	NT
Ch(1)	CH/RG	NNMC	P	1	0	NT	NT	2	1	NT	NT
Ch(2)	CH/RG	In house	P	NT	3	NT	NT	NT	3	NT	NT
Ge	GE	In house	P	2	3	NT	NT	2	3	NT	NT
Cr^a	CROM1	In house	P	2	1	NT	NT	2	2	NT	NT
Tc^a	CROM2	In house	P	2	1	NT	NT	2	1	NT	NT
Kn^a	KN1	In house	P	0	0	NT	NT	0	0	NT	NT
I	I1	Gamma	P	2	2	4	4	2	1	4	4
i	I2	In house	P	0	0	NT	NT	0	0	NT	NT
P	GLOB1	In house	P	3	2	3	NT	2	2	3	NT
$PP1P^k$	GLOB	In house	P	1	1	NT	NT	1	2	NT	NT
Lan	Lan	In house	P	3	3	NT	NT	3	3	NT	NT
Jr^a	Jr^a	In house	P	1	3	NT	NT	2	3	NT	NT

NT = not tested; O1 = first test of O cells; WRAMC = Walter Reed Army Medical Center; W = weak; O2 = second test of O RBCs; NNMC = Navy National Medical Center; P = polyclonal antibody; M = monoclonal antibody.

with the donor's RBCs (Interstate Blood Bank, Memphis, TN, USA) or autologous serum from the RBC donor. The RBCs were stored for up to 14 days at 4 °C if not cultured immediately.

P. falciparum was cultured using a modification of a described method (Ifediba & Vanderberg, 1981). To initiate the culture, a 1·5 mL aliquot of 50% RBC suspension was added to each flask along with a thawed suspension of cryopreserved *P. falciparum* organism (strain FVO). The culture was maintained at 37 °C in 5% carbon dioxide, 5% oxygen and 90% nitrogen. A control flask was prepared and maintained under the same conditions without the addition of *P. falciparum*. The culture medium was changed daily by aspirating the supernatant and replacing it with fresh RPMI complete medium.

At the end of the culture period, aliquots were taken to determine parasitaemia levels. To blind the laboratory performing the RBC phenotyping assays, malaria-infected and noninfected cultures were randomly labelled A or B by the culturing laboratory before samples were sent to the RBC antibody testing laboratory.

Assessment of parasitaemia levels

Blood films were used to determine parasitaemia levels in the malaria-infected cultures. Films were prepared by aspirating 0·5–1·0 mL of the cultured RBCs with a sterile pipette, transferring the sample to an Eppendorf tube and centrifuging it for 15 s. The supernatant was aspirated, and the pellet was resuspended and mounted on a slide by spreading into a thin film and air-drying at room temperature. The films were fixed with methyl alcohol and stained with 5% Giemsa solution. The slides were then air-dried and examined using light microscopy at 100× magnification to determine the parasitaemia levels.

Preparation of samples for phenotyping

Malaria-infected and noninfected culture samples were centrifuged at 800 *g* for 5–10 min (Sero-fuge II, Clay Adams Division of Becton Dickinson, Parisippany, NJ, USA), and the supernatant was removed and stored at 4–6 °C. The packed RBCs remaining in each tube were washed three times with 0·9% isotonic saline and packed after the final wash. An aliquot was saved for RBC counting and staining with FITC-labelled antibodies and analysis by flow cytometry. The remainder was resuspended in 0·9% saline to a 2–5% suspension for phenotyping by haemagglutination. A direct antiglobulin test (DAT) was performed on both malaria-exposed and unexposed samples prior to the phenotyping to determine if IgG or complement was coating the RBCs, which could cause false-positive results with antisera that required the use of antiglobulin reagents for phenotyping.

RBC phenotyping by haemagglutination

The donor RBCs were phenotyped before and after culture. Blinded samples were tested in parallel with each antiserum according to the manufacturers' directions or established guidelines for in-house antisera. All commercial antisera were tested against known antigen-positive and -negative control RBCs. For antisera specific to high-incidence antigens, only known antigen-positive control RBCs were tested. Cultured RBCs were tested with a second source of antiserum when a difference was noted in reactivity between malaria-infected and noninfected samples.

Testing RBCs with lectins

RBCs were tested with lectins according to the manufacturers' instructions. The carbohydrate specificity and common name for each lectin tested are listed in Table 2. Ficin-treated RBCs were used as a positive control for *Glycine soya*. RBCs used as positive and negative controls for the other lectins were group A_1 and B, respectively, for *Dolichos biflorus,* N-positive and N-negative, respectively, for *Vicia graminea*, and group O and A_1, respectively, for *Ulex europaeus*.

Testing RBCs with polybrene

The polybrene test is a rapid screening test for RBC sialic acid deficiency. A 1% polybrene solution was prepared by adding 1 g of polybrene (hexadimethrine bromide) to 100 mL of phosphate-buffered saline (PBS) at a pH of 7·0. Two drops of 1% polybrene solution were added to one drop of (2–5%) RBCs and incubated at room temperature for 5 min. The tubes were gently rocked and observed for macroscopic aggregation, which is a negative test (sialic acid present). A sample of each of the RBCs tested was treated with ficin and tested in parallel as a positive control (no aggregation).

Analysis of RBCs by flow cytometry

Flow cytometry analysis was performed on *P. falciparum*-exposed and unexposed RBCs using FITC-conjugated mouse antihuman monoclonal antibodies to CD35, CD47, CD55 and CD59 as well as isotype controls. RBCs were washed three times with 0·9% PBS prior to incubation with the antibodies. RBCs at a concentration of $10^6\,mL^{-1}$ were stained with each

Table 2. Comparison of lectin and polybrene tests of RBCs with and without exposure to *Plasmodium falciparum*

				Exposed cells				Naïve cells			
Lectin	Common name	Carbohydrate specificity	Antigen specificity	O1	O2	A_2	B	O1	O2	A_2	B
Dolichos biflorus	Horsegram	α-D-GalNAc >> α-D-Gal	A, Tn, Cad	0	0	0	0	0	0	0	0
Ulex europaeus	Gorse/furze	α-L-Fuc	H, Th, Tx, Nor	4	3	2	0	4	3	2	0
Vicia graminea	N/A	O-linked Galβ(1-3)-GalNAc	N	0	0	0	0	0	0	0	0
Arachis hypogaea	Peanut	D-Galβ(1-3)-GalNAc > β-D-Gal	T, Tk, Th, Tx	0	0	0	0	0	0	0	0
Salvia sclarea	Clary	α-D-GalNAc	Tn	0	0	0	0	0	0	0	0
Salvia horminum	Clary	α-D-GalNAc > β-D-GalNAc	Tn, Cad	0	0	0	0	0	0	0	0
Glycine max (soya)	Soybean	α-D-GalNAc > β-D-GalNAc >α-D-Gal	A1, A2, B, T, Tn	0	0	0	0	0	0	0	0

antibody. The RBCs and antibodies were incubated in the dark at 4 °C for 30 min, washed and fixed with 2% formaldehyde prior to analysis by flow cytometry. At least 100 000 cells were analyzed with a flow cytometer (EPICS Elite ESP, Beckman Coulter Corporation, Hialeah, FL, USA).

RESULTS

RBC phenotyping following P. falciparum culture

RBC phenotypes and DAT results were compared before and after culture with *P. falciparum*. RBCs from three donors were tested: one each with groups O, A and B RBCs. Blood from the donor with group O RBCs was studied twice; results of the first study are indicated by O1 and the second by O2 (Table 1). The proportion of RBCs infected with *P. falciparum* ranged from 9·5 to 13·0% (Table 3).

The DAT was negative for all RBCs, indicating that no antibody or protein was attached to the RBCs that could cause false-positive results in the haemagglutination phenotyping assay. No changes were found after culture with *P. falciparum* for the following antigens: A, B, M, N, s, P1, D, C, E, c, e, C^w, K, Kp^a, Le^a, Fy^a, Fy3, Fy6, Jk^a, Jk^b, Wr^a, Wr^b, Co^a, LW, Ge, Tc^a and Lan (Table 1). A decrease of one grade of reaction strength was noted with the following antigens: U, En^a, Kp^b, Cr^a and Jr^a with some of the *P. falciparum*-exposed cells. An increase of one grade of reaction strength was noted with the following antigens: S, k, Le^b, Di^b, I and P with a few of the *P. falciparum*-exposed cells. An increase of one grade of reaction strength was found with anti-Fy^b with O cells both times they were tested (O1 and O2), but there was no change in the reaction strength of anti-Fy^b between exposed and unexposed groups A and B RBCs. An increase in reaction strength of

Table 3. Proportion of red blood cells (RBCs) infected with trophocyte and ring forms of *Plasmodium falciparum*

		P. falciparum-infected RBCs (%)		
Study No.	Blood group	Total	Trophocytes	Ring forms
1	O*	13·0	10·0	3·0
2	O†	12·2	6·4	5·8
3	A	11·0	8·5	2·5
4	B	9·5	7·0	2·5

*First study of O RBCs (O1). †Second study of O RBCs (O2).

one anti-Lu^b was noted the first time *P. falciparum*-exposed O cells were tested, but when *P. falciparum*-exposed and unexposed O RBCs were tested with an additional anti-Lu^b and the O cells tested a second time, no changes in reaction strength were noted. A decrease in reaction strength was also found when testing exposed O RBCs with anti-Ch, but no changes were found when testing O RBCs exposed to *P. falciparum* a second time with a second source of anti-Ch. None of the above changes was considered significant.

Analysis of RBCs with lectins, polybrene and flow cytometry

Haemagglutination testing with lectins revealed no changes between *P. falciparum*-exposed and unexposed cells (Table 2). The reactions of polybrene to *P. falciparum*-exposed and unexposed RBCs from the groups O, A and B donors were the same (data not shown). There was no difference in the reactions of CD35, CD47, CD55 or CD59 antibodies with exposed and unexposed RBCs (data not shown).

DISCUSSION

P. falciparum does not appear to alter the expression of the classified immunogenic antigens on RBC membranes in our *in vitro* culture system, suggesting that severe *P. falciparum* anaemia is not owing to antibodies binding to neoantigens or crypt antigens on uninfected RBCs. RBCs were indirectly tested for neoantigens by assessing changes in blood group antigens. Because none of the antigens was affected, it is likely that *P. falciparum* does not change antigens on uninfected RBCs. RBCs were tested directly for the presence of crypt antigens using lectins and none was detected.

Although the method used in this study measured neoantigen formation indirectly, this method has previously been used to show that biotinylation of RBCs creates neoantigens. *In vitro* studies by Cowley and colleagues found that following biotinylation, anti-Di^b and anti-LW^a no longer agglutinated antigen-positive RBCs and a monoclonal anti-A agglutinated group B RBCs (Cowley *et al*., 1999). Later, Cordle and colleagues showed that following the infusion of biotinylated RBCs to healthy individuals, three of 20 developed transient antibodies that reacted with biotinylated RBCs but not unbiotinylated RBCs (Cordle *et al*., 1999).

These studies do not exclude the possibility that the antigens of *P. falciparum*-infected RBCs are changed. The results of the haemagglutination and lectin-binding assays used in this study best reflect the entire RBC population. Approximately, 10% of cells were infected, and differences in antigen expression in this proportion of cells would not probably be detected. It is also possible that the *in vivo* effects of *P. falciparum* are different than the *in vitro* effects on RBC antigens. Further studies that assess antigen expression on RBCs from patients infected with *P. falciparum* particularly those with severe anaemia would be worthwhile.

Waitumbi and colleagues found that the RBC expression of CD35, CD55 and CD59 changed in children with severe *P. falciparum* anaemia (Waitumbi *et al*., 2000). The RBC expression of CD35 and CD55 decreased, and the expression of CD59 increased. We found no difference in the expression of these antigens between *P. falciparum*-cultured and control RBCs, suggesting that changes in the expression of these antigens are not owing to *P. falciparum* alone, but may be the result of interactions among RBCs, antibodies, complement and the recticuloendothelial system.

Mouse models have shown that RBCs that lack CD47 are rapidly cleared from the circulation by splenic macrophages (Oldenborg *et al*., 2000). A decrease in the RBC expression of CD47 during *P. falciparum* infection would contribute to severe malarial anaemia, but no changes were found in CD47 expression on *P. falciparum*-cultured RBCs.

Cultured RBCs were not assessed for the expression of *P. falciparum* proteins, but soluble *P. falciparum* proteins are present in the plasma of infected individuals, and it is possible that *in vivo* these proteins bind to uninfected RBCs, and these antigen-coated RBCs are the target of antibodies directed to *P. falciparum*. It is also possible that antibodies bound to *P. falciparum* antigens expressed by infected RBCs activate complement components leading to 'innocent bystander' clearance of uninfected RBCs.

In conclusion, we could find no evidence that *P. falciparum* creates neoantigens or disrupts RBC antigens. Studies that assess the specificities of antibodies eluted from the RBCs from children with severe *P. falciparum* anaemia would be worthwhile.

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