Surface Antigen Expression on *Plasmodium falciparum*-infected Erythrocytes Is Modified in α- and β-Thalassemia

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Summary

In an attempt to determine the mechanism whereby thalassemia in its milder forms may protect against malaria, we have examined the expression of neoantigen at the surface of *Plasmodium falciparum*-parasitized thalassemic red cells. Neoantigen expression was estimated by measurement of antibody bound after incubation in serum from adults living in a malaria-endemic area, using a quantitative radiometric antiglobulin assay. We found that *P. falciparum*-parasitized α- and β-thalassemic red cells bind greater levels of antibody from endemic serum than controls: mean binding ratios (± SE), respectively, for α- and β-thalassemia compared with controls were 1.69 ± 0.12 and 1.23 ± 0.06 on a cell for cell basis, and 1.97 ± 0.11 and 1.47 ± 0.08 after a correction for surface area differences. Binding of antibody increased exponentially during parasite maturation. In addition, we found a small but significant degree of binding of naturally occurring antibody to parasitized red cells, the extent of which was also greater in thalassemia. The apparent protective effect of thalassemia against malaria may be related to enhanced immune recognition and hence clearance of parasitized erythrocytes.

Epidemiological studies suggest that the major inherited red cell disorders have attained high frequencies in malarious areas by conferring protection in their mild or heterozygous forms against *P. falciparum* malaria (1). Recent work has provided strong support for this malaria hypothesis in relation to α-thalassemia (2). Previous in vitro studies have suggested that enhanced susceptibility to oxidant stress in the parasitized variant cells may be the cellular mechanism leading to malaria resistance in heterozygous β-thalassemia and glucose-6-phosphate dehydrogenase (G6PD)[1]-deficient cells (3), although data in support of this mechanism are extremely limited for α-thalassemia. Red cells from individuals with hemoglobin H disease (the severe form of α-thalassemia in which three α genes are deleted) and homozygous β-thalassemia fail to support normal in vitro growth of *P. falciparum*. However, the relative advantage required to generate selection pressure must operate in the milder (one or two gene deletion) forms of α-thalassemia and in heterozygous β-thalassemia, and significant growth impairment has not been observed in these conditions under standard laboratory conditions (4–6). Others have reported that *P. falciparum*-infected α-thalassemic erythrocytes may be more susceptible to phagocytosis than normal parasitized cells (5).

The precise mechanisms underlying clinical immunity to malaria, and the antigens involved, are not yet understood. However, the maturation of asexual blood stages of *P. falciparum* is associated with the appearance of neoantigens on the surface of the parasitized red cell (7), and theoretical considerations indicate that this site, with its prolonged exposure to the immune system, may form a good target for immune responses (8). Animal and in vitro studies have demonstrated a range of potential antiparasite effector mechanisms that could be promoted by antibodies to the surface of parasitized erythrocytes, including opsonisation (9), complement-mediated lysis (10), antibody-dependent cytotoxicity (11), and inhibition of sequestration of infected cells (12).

It has recently been hypothesized that a contribution from the immune response may be implicated in the protective effect of the hemoglobinopathies against malaria (13). Furthermore, we have obtained evidence that favors a role in clinical immunity to malaria for the humoral response to parasite-induced neoantigens in humans living in an area highly endemic for *P. falciparum* (14). We therefore set out to investigate whether modification of neoantigen expression may contribute to the protective effect of thalassemia against malaria.

[1] *Abbreviations used in this paper:* AS, autologous serum; ES, endemic serum; G6PD, glucose-6-phosphate dehydrogenase; MCV, mean cell volume; SA, surface area; WB, wash buffer.

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Materials and Methods

Parasite Culture. *P. falciparum* strain IT (Brazil), a knob-positive line, was maintained in continuous culture by conventional methods (15) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with glucose (to 22 mM), L-glutamine (to 2 mM), Hepes (35 mM; Gibco Laboratories), and 10% human serum. Parasites were regularly stage synchronized by sorbitol lysis (16) and gelatin flotation (17).

Red Cells. Whole blood was collected into acid citrate-dextrose (1:10; vol/vol), stored at 4°C, and used within 48 h of collection. For each thalassemic sample, a normal sample was taken as control at the same time and handled identically thereafter. Samples were taken from five individuals with two gene deletion forms of α-thalassemia (heterozygous α^0 thalassemia [$--/\alpha\alpha$] or homozygous α^+ thalassemia [$-\alpha/-\alpha$]), the genotypes having been determined previously by DNA analysis (18); from three with β-thalassemia trait, as determined by hematology and HbA_2 analysis, and from eight normal controls. Sickle cell trait and G6PD deficiency were excluded in all samples by hemoglobin electrophoresis and G6PD estimation. Mononuclear cells were removed by centrifugation using Lymphoprep (Nycomed, Birmingham, UK), and the red cells were washed in RPMI 1640.

Reagents. The wash buffer (WB) was PBS (Oxoid, Basingstoke, UK) with 0.5% BSA (Sigma Chemical Co., St. Louis, MO).

Antisera. Endemic serum (ES) was pooled from six Gambian adults living in a highly malaria-endemic area and stored at −20°C. Before use, the serum was extensively absorbed against normal washed group A and B rhesus-positive red cells to remove ABO and rhesus antibodies. ^{125}I-anti-IgG (sheep anti-human) and normal sheep serum were used in the initial series of experiments on α-thalassemic cells. Insufficient sheep anti-IgG was available for use in the subsequent series on β-thalassemic cells, for which ^{125}I-labeled rabbit anti-human IgG and rabbit normal serum were used. All sheep and rabbit reagents were prepared as previously described, and adsorbed extensively against normal human red cells before use. All serum used was heat inactivated (56°C for 30 min). The number of ^{125}I-IgG molecules binding to one molecule of IgG at the red cell surface was previously calculated to be seven for the sheep anti-human antibody, and five for the rabbit anti-human antibody (19).

Microagglutination Assay. 5 μl of parasitized red cell pellets (parasitemia 5–15%) was incubated in doubling dilutions of immune serum. Agglutination of parasitized cells, visualized under uv light after specific uptake of ethidium bromide, was assessed blind by two observers, and graded absent (−) to macroscopic (++++), on the basis of agglutinate size (20).

Estimation of IgG Bound to Parasitized Cells. The method was adapted from that described by Merry et al. (19). Parallel cultures of *P. falciparum* were set up in thalassemic and control red cells, using equal inocula of gelatin-purified schizonts. These cultures were established in RPMI growth medium supplemented as described above, using 10% AB serum. The parasitemias (percent = parasites/100 red cells) were calculated from Giemsa-stained blood smears (1,000 cells counted), and maturation counts (percent schizonts) were obtained by determining the proportion of parasites in singly infected cells to have undergone nuclear division. After one parasite life-cycle, a fraction rich in trophozoites and schizonts (layer) was obtained by gelatin flotation, and the sedimented cells (pellet) were retained as a control. The layer was diluted where necessary with washed uninfected red cells of the same type (mean parasitemia [±SE] for experiments 1–5 was 75.2 ± 2.1, and 43.3 ± 2.7 for experiments 6–10). The pellet contained virtually parasite-free cells (parasitemia <<1%), which had been subjected to identical conditions as the parasite-rich layer, and was therefore an appropriate control. Both fractions were washed three times in WB, and then incubated in either ES or WB for 30 min at 37°C. After washing three times in WB, triplicate 5-μl pellets were prepared for each sample, and to each of these, 100 μl of a 1:5 solution of sheep or rabbit serum and 100 μl ^{125}I-anti-IgG (20 μg/ml) were added. After further incubation on a shaking platform for 30 min at 37°C, the samples were washed a further three times in PBS. The pellets were resuspended in 120 μl of WB. Of this, 10 μl was diluted 1:10^4 in Isoton (Coulter Immunology, Hialeah, FL) for cell count determination using a counter (Coulter Immunology); and 100 μl was spun through oil (4:1 di-*n*-butyl/dinonyl phthalate; Sigma Chemical Co.) at 13,000 rpm for 15 s in a 0.4-ml polypropylene tube (Sarstedt, Leicester, UK). The red cell pellet was cut off the bottom of the centrifuge tube and counted in a gamma counter. The following formula was used to calculate IgG bound (molecules/cell): IgG bound = (cpm/sra) $(6 \times 10^3/n)$; where cpm = counts/min/100-μl sample; sra = specific radioactivity of ^{125}I-IgG in counts/min/μg IgG; and n = cell count/100 μl $\times 10^{-7}$.

To estimate IgG bound to parasitized cells, values for specific IgG bound (molecules/cell) for nonparasitized cells (pellet^{ES} − pellet ^{WB}) were subtracted from the respective parasitized fraction results (layer^{ES} − layer^{WB}), and the result was divided by parasitemia ($\times 10^{-2}$). The value for nonparasitized cells was then added, providing a final figure for total IgG bound to parasitized cells in molecules/cell.

The thalassemic cells were consistently smaller than controls, as reflected by significant differences in mean cell volume as measured by a counter (ZM analyzer; Coulter Immunology) (Table 1). To allow for this in the determination of IgG bound, the surface area of cells was estimated from the mean cell volume using the formula: SA = 44.8 + 1.17 × MCV; where SA = surface area (μ^2); and MCV = mean cell volume (fl), derived from published red cell measurements (21). IgG bound was then calculated per 100-μ^2 surface area. Results for thalassemic cells and controls were compared using a paired Student's t test (two tailed).

Binding of Naturally Occurring IgG. Antibodies are present in normal human serum and bind to modified components at the surface of normal red cells, and the target antigens involved may be increased by malaria infection (22). To estimate binding of such naturally occurring IgG to parasitized red cells, and to make a direct comparison within one experiment of IgG bound after incubation in ES and WB, the above procedure was repeated in parasitized normal cells with the addition of samples incubated in serum from the red cell donor. This autologous serum (AS) was diluted to 50% with RPMI 1640 before use.

Stage-related Variation in IgG Binding. The relationship between IgG bound and parasite maturation was determined by estimating IgG binding after incubation in ES at six hourly intervals during a complete parasite life-cycle. Parasites were tightly synchronized by regular alternating sorbitol lysis and gelatin flotation over a 2-wk period. The experimental culture was then set up at 30% parasitemia in identical flasks containing 50 μl of packed red cells in 20 ml of medium. At six hourly intervals, the cells in a flask were washed and triplicate pellets incubated in ES or WB, followed by estimation of IgG bound as described above. The following controls were included: (*a*) nonparasitized cells, cultured in a parallel flask and treated identically (as an estimate of background values); and (*b*) nonparasitized cells after 12 h incubation in culture supernatant derived from the parasite culture during the reinvasion period, which preceded the cycle studied (to account for the possibility that during schizont rupture and reinvasion, antigens released into growth medium might associate with nonparasitized cells, promoting IgG binding to these cells).

Results

The characteristics of the red cells used in the experiments are shown in Table 1. The mean corpuscular volume (MCV) and calculated surface area (SA) were low for the thalassemic cells as expected, and values for α- and β-thalassemia were similar.

The semiquantitative microagglutination assay showed a greater degree of agglutination of parasitized α-thalassemic cells than controls. In one experiment, two $--/\alpha\alpha$ samples at 14% parasitemia were scored as +++ agglutination, while controls were scored as + only, when incubated in ES at a titre of 1 in 20 (data not shown). In a second experiment at the same titre of ES, $-\alpha/-\alpha$ red cells at 4% parasitemia showed moderate (++) agglutination, while there was no agglutination of parasitized control cells.

The results of the antiglobulin assay, in which IgG bound to parasitized thalassemic cells after incubation in ES was estimated, are shown for α-thalassemia (Fig. 1 *a*) and for β-thalassemia (Fig. 1 *b*). Parasitized thalassemic red cells consistently bound more IgG than controls. The mean binding ratios (± SE) for α- and β-thalassemic cells compared with controls were 1.69 ± 0.12 and 1.23 ± 0.06, respectively, on a cell for cell basis (differences between thalassemic cells and controls were statistically significant at $p \leq 0.02$). When calculated per unit surface area, those ratios were 1.97 ± 0.11 and 1.47 ± 0.08, respectively.

Infected cells incubated in WB also appeared to bind significant amounts of IgG (mean, 12.7% of that bound in ES) when compared with uninfected cells. This was presumed to represent specific naturally occurring antibody derived from human serum in the growth medium that had bound during parasite culture preceding the assay. Moreover, antibody bound to such WB-incubated cells was consistently greater for thalassemic cells when compared with controls (Fig. 2, *a* and *b*). For α- and β- thalassemic cells, the mean binding ratios (±SE) of cells incubated in WB compared with controls were 1.29 ± 0.14 and 1.31 ± 0.12, respectively, on a cell for cell basis,

Table 1. *Characteristics of Blood Samples*

Cell type	No.	MCV	Surface area*
		mean fl	*mean* μ^2
α-Thalassemia	5‡	69.5 ± 0.8	126.1 ± 1
β-Thalassemia	3	69.6 ± 1.6	126.4 ± 1.8
Controls	8	88.8 ± 1.9	148.7 ± 2.2

* See text for derivation.
‡ Four were $--/\alpha\alpha$, one was $-\alpha/-\alpha$.

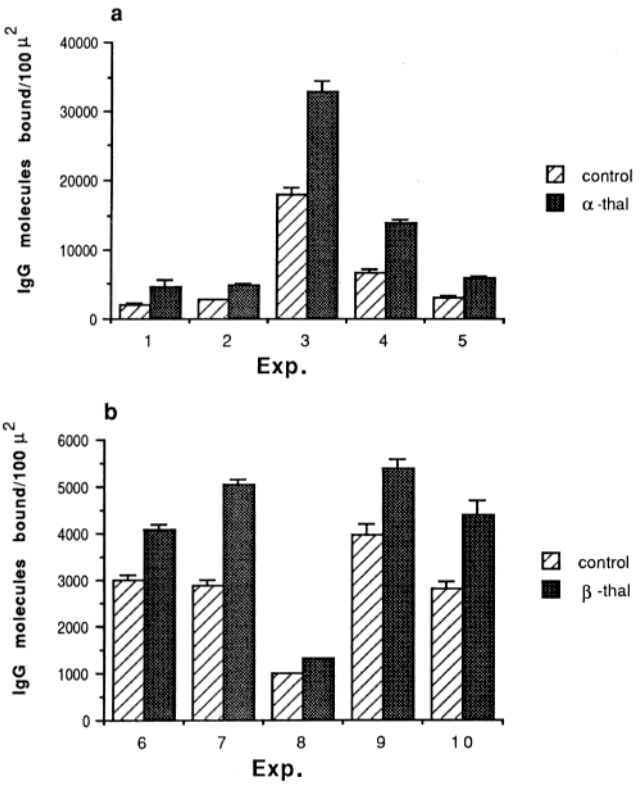

Figure 1. Antibody detected on *P. falciparum*–parasitized red cells incubated in endemic serum. IgG (molecules/100μ^2) bound to (*a*) α-thalassemic vs. control cells and (*b*) β-thalassemic vs. control cells, after incubation in pooled serum from a malaria-endemic area. Error bars show SE for triplicate values.

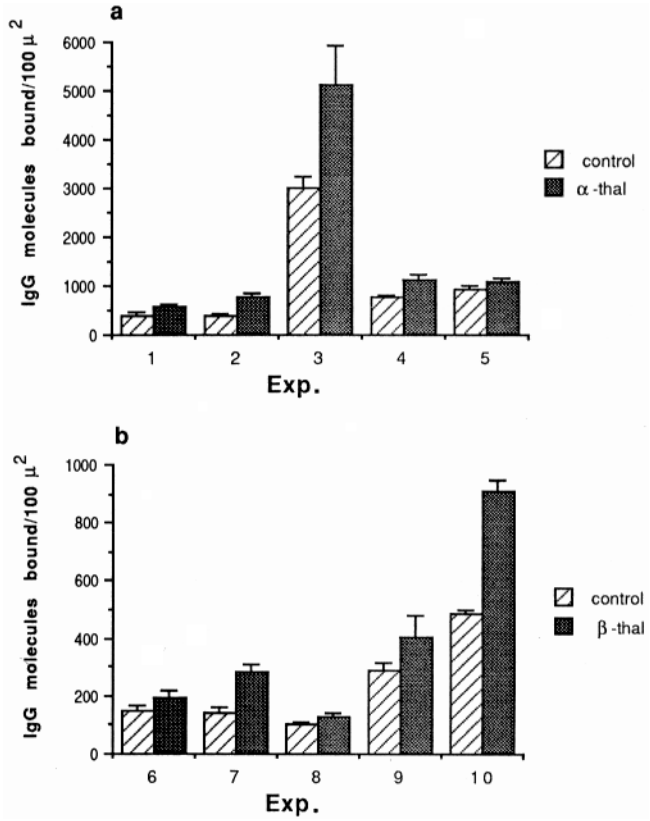

Figure 2. Antibody detected on *P. falciparum*–parasitized red cells not exposed to endemic serum. IgG (molecules/100μ^2) bound to (*a*) α-thalassemic vs. control cells and (*b*) β-thalassemic vs. control cells, after incubation in wash buffer (PBS/0.5% BSA).

Table 2. *Parasite Maturation Counts for Thalassemic Red Cells and Controls*

Exp.	Maturation count*	
	α-Thalassemia	Control
	%	
1	17	15
2	9	10
3	92	91
4	50	46
5	41	41
	β-Thalassemia	Control
6	23	19
7	15	16
8	4	5
9	22	24
10	19	18

* Percent of singly infected parasitized cells showing nuclear division.

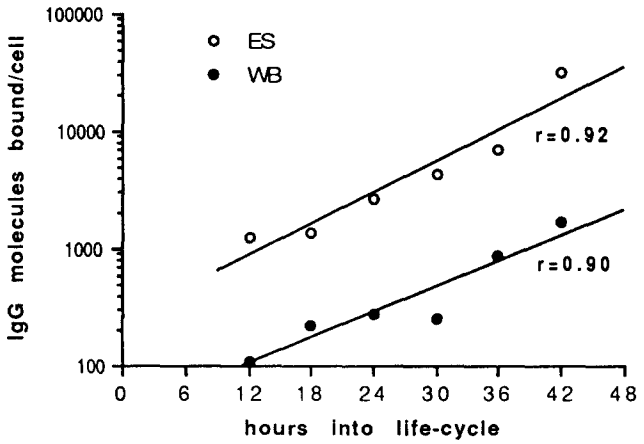

Figure 3. Stage-related variation in antibody detected on *P. falciparum*–parasitized normal red cells. Bound IgG (molecules/parasitized cell) was estimated after incubation in endemic serum (ES) or wash buffer (WB) during a complete in vitro life cycle in a tightly synchronous culture. Points represent mean values for triplicate samples (SE values were within 10% for each point). *r*, correlation coefficient for exponential fit.

and 1.58 ± 0.15 and 1.56 ± 0.14 when calculated per unit surface area (differences between thalassemic cells and controls were significant at $p < 0.02$).

To explore the binding of naturally occurring antibody, experiments were carried out on normal parasitized cells incubated in ES, WB, and AS. In one such experiment in which 10% of parasites had matured to the schizont stage, the numbers of molecules of IgG bound after incubation in AS was not significantly different from WB-incubated cells (170 ± 16 vs. 150 ± 8 molecules/parasitized cell, respectively). Values detected on ES-incubated cells in the same experiment were 3,196 ± 165 molecules/parasitized cell. Therefore, the IgG bound after incubation in AS or WB represented ~5% of values for ES samples. For experiments 1–10, the mean value for WB relative to ES was 12.7% (SE ± 1.5; range, 4.7–29.8).

To eliminate the possibility that differential parasite maturation in α- and β-thalassemic cells might affect the results, counts of maturation of parasites were carried out in all experiments. As can be seen in Table 2, parasites were capable of equally successful growth in both thalassemic and normal cell types under these conditions, as demonstrated previously (4, 6).

All experiments were performed using parasites at the pigmented trophozoite stage or later. However, although IgG binding to parasitized thalassemic cells was consistently greater than controls, the variation in absolute values with maturation count (Fig. 1) suggested that neoantigen expression may increase during parasite development. We therefore measured the change in IgG binding during parasite maturation. The results showed a steady increase from 12 h into the parasite life-cycle until well into schizont maturation (Fig. 3). This increase applied to cells incubated in both ES and WB. The nonparasitized controls showed low background levels of

Table 3. *IgG Detected on Nonparasitized Thalassemic and Control Cells after Incubation in Endemic Serum (ES) or Wash Buffer (WB)*

	α-Thalassemia	Control	p*	β-Thalassemia	Control	p*
ES (molecules/cell)	306 ± 85	516 ± 85	0.026	254 ± 84	245 ± 68	NS‡
ES (molecules/100 μ^2)	287 ± 39	345 ± 50	NS	200 ± 64	162 ± 44	NS
WB (molecules/cell)	220 ± 21	310 ± 31	NS	120 ± 26	140 ± 28	NS
WB (molecules/100 μ^2)	172 ± 16	207 ± 20	NS	112 ± 21	97 ± 19	NS

* Student's *t* test (two tailed).
‡ NS = $p > 0.05$.

binding (90–173 molecules/cell). It was not possible to determine accurately at which point in time neoantigen was first expressed, as unruptured schizonts were still present in the culture at low frequency for the first 12 h. At 18 h, when there were no residual schizonts and all the parasites had reached the early pigmented trophozoite stage, a significant amount of specific IgG was bound when compared with uninfected controls.

In contrast to infected cells, uninfected cells, obtained from the almost parasite-free pellet after gelatin flotation and exposed to the same manipulations in vitro, bound minimal IgG when incubated in ES or WB. After correlation for cell surface area, uninfected thalassemic cells bound similar amounts of IgG compared with normal red cells (Table 3). Differential binding was only observed after parasitization.

Discussion

Observations from previous in vitro studies have led to the proposal of several candidate mechanisms for the apparent malaria resistance found in the inherited red cell disorders (23, 24). The most plausible hypothesis so far in relation to thalassemia is based on observations on the differential susceptibility of parasitized normal and thalassaemic cells to oxidant stress (3). However, the high level of oxidant stress used in these experiments makes it difficult to interpret their significance. Data in support of this mechanism are not available for α-thalassemia, but in a series of experiments on single- and two-gene deletion forms of α-thalassemia, we have not observed any difference between normal and thalassemic cells under a wide range of conditions of oxidant stress (our unpublished observations).

It has been suggested that a contribution from the immune response may be implicated in the protective effect of the hemoglobinopathies against malaria (13). In cross-sectional and longitudinal studies in Gambia, we examined the relationship between several in vitro assays of the host immune response to *P. falciparum* and clinical protection against malaria. Evidence for a role in protective immunity was only demonstrable for antibodies to neoantigens on the infected erythrocyte surface (14).

Previous work has suggested that surface antigens on thalassemic cells are modified. In nonparasitized homozygous β-thalassemia, enhanced phagocytosis by mouse peritoneal macrophages has been observed, and significant levels of in situ bound IgG have been detected at the surface of these cells, but not on normal cells. This antibody was defined as naturally occurring IgG of anti-α-galactosyl specificity (25, 26). In α-thalassemia, phagocytosis by human monocytes (in the absence of immune serum) was found to be enhanced in erythrocytes infected with mature forms of *P. falciparum* (5).

Our results suggest that modification of parasite-induced neoantigens does occur on thalassemic cells, in a way that promotes the greater binding of specific antibody. By analogy with previous studies on phagocytosis of IgG-coated red cells, the degree of IgG binding to parasitized cells observed as early as the trophozoite stage in our experiments is likely to be sufficient to promote clearance (27, 28).

The exponential increase in IgG binding during parasite development was unexpected. Previous studies had shown that both agglutination and surface immunofluorescence of parasitized cells exposed to immune serum reach a plateau at ~24 h into the 48-h parasite cycle (20). Our current findings suggest that beyond mid-cycle, exposure of neoantigens and altered host membrane components continues in an exponential fashion. It remains to be seen whether this increasing antigen exposure reflects increasing export of parasite proteins during development and their incorporation into the red cell membrane, or whether other factors are responsible. Physical factors related to the enlarging intraerythrocytic parasite may modify the antigenic expression of membrane components during development.

Several novel proteins have been identified in association with the membrane of the parasitized red cell, mostly poorly characterized, and their role in immunity as yet undefined. The only one known to be present at the surface for a significant proportion of the parasite red cell life-cycle is known as PfEMP1, a protein of high molecular weight that is thought to show antigenic diversity (29). Further studies are required to identify the antigenic components responsible for the greater binding of specific antibody to parasitized thalassemic cells.

There is evidence that a second group of antigens are exposed at the red cell surface during the development of the intraerythrocytic parasite, consisting of modified host membrane components to which circulating IgG antibody is normally found. Previous work suggests that aggregated band 3 and possibly terminal α-galactosyl residues may be involved (22, 30). Our studies support stage-related exposure of antigens in this category.

In a previous study in Gambian children, significantly higher titres of antibodies to parasite-induced neoantigens, assayed by microagglutination and surface immunofluorescence, were observed among carriers of the sickle cell gene (14). This raises the important possibility that surface alterations influencing antibody binding to parasitized cells may be a feature common to all the hemoglobinopathies. We have performed preliminary studies on cells from sickle cell heterozygotes, but the interpretation of the results has been complicated by the reduced growth observed in these cells under standard in vitro culture conditions. Parasite development in these cells was retarded compared with controls under conditions of low ambient oxygen tension, as previously noted (31).

A further important possibility arising from our findings is that development of immunity to malaria may be affected by modified neoantigen expression in thalassemia. The degree of protective immunity or its rate of acquisition may be enhanced, and these possibilities can be investigated in field studies using existing in vitro assays.

In conclusion, we have demonstrated that the expression of parasite-induced surface neoantigens in α- and β-thalassemia is modified in a way that allows greater binding of specific antibody to parasitized erythrocytes. The functional significance of this observation remains to be determined, but our findings support the hypothesis that protection against malaria in thalassemia, and perhaps other inherited red cell disorders, involves immune mechanisms.

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