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

By G. A. Luzzi,* A. H. Merry,† C. I. Newbold,* K. Marsh,* G. Pasvol,* and D. J. Weatherall*

From the *University of Oxford, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU; and the †Blood Group Reference Laboratory, Radcliffe Infirmary, Oxford, OX2 6HE, England

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 milder 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.
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 αα/αα thalassemia [−/−; αα] or homozygous αα thalassemia [−/−; αα]), the genotypes having been determined previously by DNA analysis (18); from three with β-thalassemia trait, as determined by hematocrit and HbA2 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. 121I-anti-IgG (sheep anti-human) and rabbit anti-IgG (sheep anti-human) and rabbit normal serum were included: (a) nonparasitized cells, cultured in a parallel flask and triplicate pellets incubated in ES or WB followed by estimation 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 α/α 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, α/α 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 <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 2. Parasite Maturation Counts for Thalassemic Red Cells and Controls

<table>
<thead>
<tr>
<th>Exp.</th>
<th>α-Thalassemia</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maturation count* (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>92</td>
<td>91</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>β-Thalassemia</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Maturation count* (%)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>18</td>
</tr>
</tbody>
</table>

* Percent of singly infected parasitized cells showing nuclear division.

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)

<table>
<thead>
<tr>
<th></th>
<th>α-Thalassemia</th>
<th>Control</th>
<th>p*</th>
<th>β-Thalassemia</th>
<th>Control</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES (molecules/cell)</td>
<td>306 ± 85</td>
<td>516 ± 85</td>
<td>0.026</td>
<td>254 ± 84</td>
<td>245 ± 68</td>
<td>NS†</td>
</tr>
<tr>
<td>ES (molecules/100 μl)</td>
<td>287 ± 39</td>
<td>345 ± 50</td>
<td>NS</td>
<td>200 ± 64</td>
<td>162 ± 44</td>
<td>NS</td>
</tr>
<tr>
<td>WB (molecules/cell)</td>
<td>220 ± 21</td>
<td>310 ± 31</td>
<td>NS</td>
<td>120 ± 26</td>
<td>140 ± 28</td>
<td>NS</td>
</tr>
<tr>
<td>WB (molecules/100 μl)</td>
<td>172 ± 16</td>
<td>207 ± 20</td>
<td>NS</td>
<td>112 ± 21</td>
<td>97 ± 19</td>
<td>NS</td>
</tr>
</tbody>
</table>

* 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 PEMP1, 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.
We thank Dr. Yvonne Richards (Manchester Blood Transfusion Service) for providing the radiolabeled anti-IgG antibodies, Dr. D. Higgs for the α-thalassemia genotyping, and Dr. J. B. Clegg for helpful comments.

This work was supported by the Wellcome Trust and Medical Research Council of Great Britain.

Address correspondence to Dr. G. A. Luzzi, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, England.

Received for publication 2 October 1990.

References

29. Howard, R.J. 1988. Malarial proteins at the membrane of Plasmodium falciparum-infected erythrocytes and their involvement with...
in cytoadherence to endothelial cells. Prog. Allergy. 41:98.
