Enhanced phagocytosis of ring-parasitized mutant erythrocytes. A common mechanism that may explain protection against falciparum-malaria in sickle-trait and beta-thalassemia-trait

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High frequency of erythrocyte (RBC) genetic disorders such as sickle-cell trait, thalassemia-trait, homozygous Hb-C and G6PD-deficiency in regions with high incidence of *Plasmodium falciparum* malaria, and case-control studies support the protective role of those conditions. Protection has been attributed to defective parasite growth or to enhanced removal of the parasitized RBC. We suggested enhanced phagocytosis of rings, the early intraerythrocytic form of the parasite, as an alternative explanation for protection in G6PD-deficiency (Cappadoro et al. Blood. 1998;92:2527-2534). We show here that *P. falciparum* developed similarly in normal RBC and in sickle-trait, beta- and alpha-thalassemia trait, and HbH RBC. We also show that membrane-bound hemichromes, autologous IgG and complement C3c fragments, aggregated band-3 and phagocytosis by human monocytes were remarkably higher in rings developing in all above mutant RBC except alpha-thalassemia trait. Phagocytosis of ring-parasitized mutant RBC was predominantly complement-mediated and very similar to phagocytosis of senescent or damaged normal RBC. Trophozoite-parasitized normal and mutant RBC were phagocytosed similarly in all conditions examined. Enhanced phagocytosis of ring-parasitized mutant RBC may represent the common mechanism for malaria protection in non-immune individuals affected by widespread RBC mutations, while individuals with alpha-thalassemia trait are likely protected by a different mechanism.
**Introduction**

High frequency of hemoglobinopathies such as sickle-cell trait, thalassemia-trait, homozygous Hb-C and Hb-E; of glucose-6-phosphate dehydrogenase (G6PD)-deficiency and Southeast Asian ovalocytosis (SAO) in regions with past or present high incidence of *Plasmodium falciparum* malaria, and case-control studies support the protective role of those conditions (see ref. 1-4 for reviews). Protection has been attributed in some studies to defective invasion or growth of the parasite in the mutant erythrocytes (RBC)\(^5,7\) while other studies found normal parasite invasion and growth.\(^8,9\) Since the original proposal by Haldane,\(^10\) microcitemia and increased osmotic resistance, enhanced oxidant radical production due to unpaired globin chains, increased sickling, ionic unbalances or membrane rigidity or molecular defects in band 3 have been suggested as underlying mechanism explaining impaired growth of the parasite in the mutant RBC.\(^1,4,11-13\) Other studies have found increased deposition of nonspecific autologous antibodies on malaria-parasitized mutant RBC and suggested phagocytic elimination as the possible mechanism of protection in non-immune subjects.\(^14-16\) Recently, we found that several strains of *P falciparum* developed similarly in normal and G6PD-deficient RBC.\(^9\) However, ring-parasitized G6PD-deficient RBC bound more opsonins such as autologous IgG and complement C3c fragments, and were phagocyted more intensely than their normal counterparts.\(^9\) We suggested enhanced ring phagocytosis as an alternative explanation for malaria protection in G6PD-deficient individuals, and discussed why removal of early parasite forms could be advantageous to the host.\(^9\)

We show here that *P falciparum* invaded and matured similarly in normal and mutant RBC (heterozygous sickle-cell anemia, HbAS; heterozygous beta-thalassemia, beta-thal trait; homozygotes for alpha-plus thalassemia, alpha-thal trait; compound heterozygotes for alpha-zero and alpha-plus thalassemia, HbH disease) up to the third cycle of invasion. We also show that membrane-bound hemichromes, aggregated band 3, autologous IgG and complement C3c fragments, and phagocytosis by adherent human monocytes were remarkably higher in all above mutants except alpha-thal trait. Enhanced phagocytosis of ring-parasitized mutant RBC was mostly complement-mediated and very similar to phagocytic recognition and removal of senescent or oxidatively damaged normal RBC.\(^17-19\) Enhanced phagocytic removal of early parasite forms may thus represent a common mechanism for malaria protection in widespread RBC mutations.

**Materials and methods**

**Materials**

Monoclonal antibodies against human RBC membrane proteins band 3, rabbit anti-human IgG second antibodies, rabbit anti-human C3c second antibodies, mouse anti-rabbit second antibodies (all second antibodies conjugated to alkaline phosphatase), beta-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), N-ethyl maleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin, and sodium dodecyl sulfate (SDS), bovine serum albumin and Tween-20 were from Sigma (St Louis, MO). Octaethylene glycol mono-n-dodecyl ether (C\(_{12}\)E\(_8\)) was from Nikkei Chemical Co. (Tokyo, Japan) or Sigma. Eosin-5-maleimide was from Molecular Probes (Eugene,
OR). Sepharose CL-6B, Protein A-Sepharose beads and Percoll were from Pharmacia Biotech (Uppsala, Sweden). Sterile plastics were from Costar (Cambridge, MA). Diff-Quik parasite stain was from Baxter Dade AG (Dudingen, Switzerland). All other reagents were purchased from common commercial sources.

Preparation of RBC from normal and mutant subjects, cultivation of P. falciparum and stage-separation of parasitized RBC

Control RBC were obtained from hematologically healthy subjects aged 20-55 years. Controls were staff members or blood donors from the local blood bank. All controls had normal hematological parameters and normal G6PD activity. The following mutant RBC were obtained from healthy subjects of both sexes aged 20-58 years: heterozygous beta-thalassemia (Codon beta-39 (C→T)/N), beta-thal trait; alpha-thalassemia (homozygotes for alpha plus-thalassemia, -α^3.7/-α^3.7), alpha-thal trait; compound heterozygotes for alpha zero and alpha plus-thalassemia, HbH; and carriers for HbAS (Codon beta-6 (A→T)/N), HbAS. In all cases informed consent was obtained and the study protocol was approved by the Torino University Medical School Ethical Committee. Standard hematologic parameters and the genotype of the mutant subjects are shown in Table 1. HbAS subjects were of African descent, while beta- and alpha-thalassemia subjects were of Sardinian origin. Molecular diagnosis of beta-thalassemia, alpha-thalassemia and HbAS was carried out by PCR methodology. None of the control or mutant subjects was G6PD-deficient. Blood from normal and mutant individuals, anticoagulated with citrate-phosphate-dextrose with adenine was used immediately after withdrawal. RBC were isolated from plasma and white blood cells by 80% Percoll gradient in phosphate buffered saline (PBS) centrifugation and 3 washings in wash medium (RPMI 1640 medium containing 25 mmol/L Hepes, 20 mmol/L glucose and 32 mg/L gentamicin, pH 6.80). Most studies were performed with P. falciparum strain Palo Alto. Additional studies were performed with strains H3BA, 3D7A, 1776 and FCR-3. All strains were mycoplasma-free. Parasites were cultivated at 1% hematocrit, synchronized and separated as described. Culture medium (RPMI 1640 medium with Hepes and glutamine) was supplemented with 10% fresh autologous serum, 20 mmol/L glucose and 0.8 mmol/L adenine (final concentration), and changed daily to ensure optimal parasite growth. In all studies, nonparasitized normal and mutant controls were kept in culture under the same conditions as parasitized RBC. Indeed, the standard culture conditions at high CO2 concentration and slightly lower pH-values in RBC had been previously shown to induce changes in normal controls that led to increased phagocytic rate. For all studies, parasitemia of inoculum was adjusted to 20% schizont-parasitized RBC (schizonts) for isolation of rings and to 5% schizonts for isolation of trophozoites and schizonts. Fourteen-18 hours after inoculum (rings), 34-38 hours (trophozoites) and 40-44 hours after inoculum (schizonts), rings, trophozoites and schizonts were separated on Percoll-mannitol gradients. The parasitemia was usually 35-45% rings, >95% trophozoites and >95% schizonts. Except for the invasion and maturation experiments, parasitized RBC were isolated during the first cycle of parasite growth. The total number of nonparasitized and parasitized RBC was counted electronically by Coulter Counter. To assess total parasitemia and relative contribution of rings, trophozoites and schizonts, slides were prepared from cultures at indicated times, stained with Diff-Quik parasite stain and 400-1000 cells examined microscopically.

Assessment of parasite invasion and maturation

Inoculum was performed mixing separated normal schizonts with normal or mutant RBC. Parasitemia of inoculum was adjusted to 4%, final hematocrit of inoculum was 0.5%. Invasion: ratio between ring parasitemia measured 14-18 hours
after inoculum and inoculum parasitemia (first cycle invasion), or ratio between ring parasitemia measured 62-66 hours after inoculum and trophozoite parasitemia measured 40-44 hours after inoculum (second cycle invasion) or ratio between ring parasitemia measured 88-92 hours after inoculum and trophozoite parasitemia measured 62-66 hours after inoculum (third cycle invasion). Maturation: ratio between trophozoite parasitemia measured 34-38 hours after inoculum and ring parasitemia measured 14-18 hours after inoculum (first cycle maturation), or ratio between trophozoite parasitemia measured 82-86 hours after inoculum and ring parasitemia measured 62-66 hours after inoculum (second cycle maturation), or ratio between trophozoite parasitemia measured 102-106 hours after inoculum and ring parasitemia measured 82-86 hours after inoculum (third cycle maturation). Nonparasitized and parasitized RBC were counted electronically. Total parasitemia and relative contribution of rings, trophozoites and schizonts were assessed as described in the preceding section.

Opsonization of RBC
Freshly separated nonparasitized and parasitized RBC (see above) were washed 3 times in wash medium (130 mmol/L NaCl, 10 mmol/L Hepes, 10 mmol/L glucose, pH 7.4). Washed RBC were opsonized in wash medium supplemented with 33% fresh autologous serum at 33% hematocrit for 30 minutes at 37°C. Cells were then washed twice in the same medium, resuspended at 10% hematocrit and used for preparation of hypotonic membranes.

Assay of membrane-bound autologous IgG and C3c fragment
Opsonized nonparasitized and stage-separated parasitized RBC were washed 3 times in wash medium. RBC-bound IgG were measured after labeling RBC with anti-human IgG developed in goat for 1 hr at 4°C as indicated.9,23 RBC-bound complement C3c fragment was measured with anti-human complement C3c developed in rabbit used as first antibody and anti-rabbit IgG phosphatase-conjugated as second antibody. Antibodies were diluted 1:500. Labeled hypotonic membranes were solubilized in PBS containing 0.5% (vol/vol) Tween-20, and alkaline phosphatase activity was measured by visible spectrophotometry at 405 nm using nitro blue tetrazolium/bromochlorophosphate as phosphatase substrate. Bound IgG and complement C3c fragment values obtained with cultures that contained 20-25% rings, were extrapolated to 100% rings parasitemia using the following calculation: I=(TOT-N x n)/(1-n), where I=amount of bound IgG and C3c in 100% rings; TOT=amount of bound IgG and C3 in the whole culture; N=amount of bound IgG and C3c in nonparasitized RBC; n=fraction of nonparasitized RBC.9,23

Preparation and extraction of hypotonic membranes with nonionic detergent C12>E8
Standard hypotonic membranes from nonparasitized and stage-separated parasitized RBC were prepared at 0°C by hemolysis in hemolysis buffer (5 mmol/L sodium phosphate, 1 mmol/L EDTA, pH 8.0) and 2 washes. One-half mL freshly-prepared membranes was mixed as indicated23 with 1 mL of extraction buffer (130 mmol/L NaCl, 10 mmol/L Hepes, 10 mmol/L NEM, 1 mmol/L EDTA, 1 mmol/L PMSF, 0.05 U/mL aprotinin, 0.5 µg/mL leupeptin, 20 µg/mL pepstatin and 1.5% (vol/vol) nonionic detergent C12>E8, pH 7.4) at 37°C for 20 minutes under moderate shaking and then pelleted for 15 minutes at 13000 rpm in a refrigerated Eppendorf microfuge. The clear supernatant (C12>E8 extract) was immediately separated from the pellet and used for gel-filtration chromatography.
Gel-filtration chromatography of the C$_{12}$E$_8$ extract

One mL of the C$_{12}$E$_8$ extract was loaded onto a Sepharose CL-6B column (excluded volume >4000 kD molecular weight) and separated at a flow rate of 0.9 mL/minutes. The effluent was collected in 0.8 mL fractions (C$_{12}$E$_8$ fractions) as indicated.

Assay of membrane-bound hemichromes

Membrane-bound hemichromes were assayed in standard hypotonic membranes prepared as indicated (see above) by measuring absorbance at 560, 577 and 630 nm, using millimolar absorptivity values of 8.6, 6.8 and 0.92, respectively. Due to the great variability in protein content in RBC membrane preparations, membrane volume was selected as a reference and hemichromes expressed as nmol hemichrome/mL membrane. Membrane volume showed low inter-subject variability and roughly corresponded to the original RBC volume in both normal and mutant RBC.

Assay of aggregated band 3

Aggregated band 3 was assayed in the C$_{12}$E$_8$ fractions of nonparasitized and stage-separated parasitized RBC previously labelled by the band-3 specific fluorescent label eosin-5-maleimide as described. In order to quantitate the percentage of aggregated band 3, eosin-5-maleimide labelled band 3 was assayed by fluorometry in all C$_{12}$E$_8$ fractions and the fluorescence value measured in the high molecular weight fractions was normalized to the total fluorescence measured in all fractions.

Separation of human monocytes and measurement of phagocytosis

Human monocytes were prepared from freshly separated buffy coats from normal blood, and phagocytosis was quantified by measuring heme-enhanced luminescence and expressed as number of ingested RBC per monocyte as indicated.

Treatment of monocytes with anti-CR1 antibodies

Complement receptor type 1 (CR1, CD35) was blocked on monocyte membrane by the monoclonal antibody J3D3, kindly supplied by MD Kazatchkine, Paris, France. Adherent monocytes (approx. 50000 monocytes per well) were incubated at 37°C for 30 minutes in 1 mL of RPMI 1640 containing 2% (wt/vol) bovine serum albumin supplemented or not with 20 µg/mL purified anti-CR1 antibody J3D3. The concentration of J3D3 used here was maximally effective in inhibiting CR1 function as shown previously.

Statistical analysis

Significance of differences was assessed by $t$-test for paired samples.
Results

Hematologic parameters and genetic characteristics of mutant RBC

The hematologic and genetic characteristics of mutant RBC donors are presented in Table 1. To minimize influence of different genetic backgrounds, study subjects were unrelated but carried the same kind of molecular mutation.

Parasite growth in normal and mutant RBC

Invasion and maturation of *P. falciparum* (Palo Alto strain, mycoplasma-free) were measured in the first, second and third cycle of parasite growth in normal and mutant RBC with 4% parasitemia at the time of inoculum and 0.5% final hematocrit. No statistically significant difference in both parameters of parasite growth were noted during any of the three growth cycles and with any of the mutations considered (Figure 1). The experiments were repeated with four additional parasite strains (H3BA, 3D7A, 1776 and FCR-3, all mycoplasma-free) and HbAS and beta-thal trait RBC, and gave similar results (not shown)

Table 1. Hematologic parameters and genotype in mutant subjects

A) HbAS subjects

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Hb (g/dL)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>Genotype</th>
<th>HbA2 (%)</th>
<th>HbF (%)</th>
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<td>82.4</td>
<td>29.1</td>
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<td>25.5</td>
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B) Beta-thal trait subjects

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<th>MCH (pg)</th>
<th>Genotype</th>
<th>HbA2 (%)</th>
<th>HbF (%)</th>
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C) Alpha-thal trait and HbH subjects

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<th>MCH (pg)</th>
<th>Genotype</th>
<th>HbA2 (%)</th>
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Membrane binding of hemichromes, autologous IgG and complement C3c fragment; aggregated band 3 and phagocytosis in nonparasitized and ring-parasitized normal and mutant RBC

Figure 2 shows that the levels of membrane-bound hemichromes and of the other markers of membrane damage and phagocytosis were already increased at baseline conditions in nonparasitized HbAS, beta-thal trait and HbH RBC kept for comparable time periods under the same culture conditions as parasitized RBC (see Materials and Methods). The highest level of damage was in nonparasitized HbAS and HbH RBC, while alpha-thal trait RBC were indistinguishable from normal controls. Figure 3 is an overview of membrane-bound hemichromes, IgG and complement C3c; aggregated
band 3 and phagocytosis in ring-parasitized mutant RBC compared to matched ring-parasitized normal RBC. With the exception of alpha-thal trait RBC, all ring-parasitized mutant RBC had distinctly higher levels of all considered parameters. Phagocytosis was also very remarkably increased in ring-parasitized RBC in all mutations except alpha-thal trait. As expected from the base-line situation, rings developing in HbH RBC induced the highest levels of hemichromes, band 3 aggregation and C3c deposition. Heme-containing compounds were found in the fraction corresponding to the column void volume (>4000 kD molecular weight), indicating heme binding to aggregated, high molecular weight membrane components (not shown). The absorption spectrum of the heme-containing fractions corresponded to that of hemichromes. The same fractions contained aggregated band 3, which was localized by labeling hypotonic membranes of ring-parasitized RBC with specific fluorescent band-3 label eosin-5-maleimide (not shown). This chromatographic behavior showing co-elution of hemichromes and aggregated band 3 is considered as indication of hemichrome-induced clustering of band 3. The number of phagocytosed HbAS, beta-thal trait and HbH rings was close to the maximal erythrophagocytic capacity of adherent human monocytes. The role of complement as opsonin was tested in ring-parasitized beta-thal trait RBC. Figure 4 shows that abrogation of C3-mediated phagocytosis by blockage of CR1 receptor on monocytes reduced phagocytosis of ring-parasitized beta-thal trait and normal RBC by approx 80 and 94%, respectively, in agreement with previous observations. Decrease of C3-mediated phagocytosis was less pronounced in trophozoites (46 and 43% reduction in trophozoite-parasitized beta-thal trait and normal RBC, respectively), indicating that IgG- and scavenger receptors may play a more significant role in phagocytosis of mature parasite forms. Latter data are in agreement with previous observations in trophozoite-parasitized G6PD-deficient RBC. The oxidative origin of membrane damage was underscored by a partial reversion of hemichrome formation, band 3 aggregation and deposition of removal markers in normal and mutant RBC cultivated in presence of 100 µmol/L beta-mercaptoethanol, confirming previous data obtained with non-mutant parasitized RBC (not shown).

Taken together, present data indicate that membrane damage ultimately generated by the interaction of abnormal hemoglobins and the parasite and likley responsible for enhanced phagocytosis of ring-parasitized mutant RBC, was very similar to ring-parasitized G6PD-deficient RBC and to senescent or oxidatively damaged nonparasitized RBC.

Membrane binding of hemichromes, autologous IgG and complement C3c fragment; aggregated band 3 and phagocytosis in trophozoite-parasitized normal and mutant RBC

As shown in Figure 5, the differences between ring-parasitized normal and mutant RBC described before, largely vanished at later parasite development stages. Trophozoites developing in normal or mutant (HbAS and beta-thal trait) RBC were phagocytosed to the physical limit of the monocyte (approx. 10 parasitized RBC per monocyte) and differences between parasitized normal and mutant RBC appeared to vanish. Accordingly, the same generation and deposition of aggregated band 3, IgG and C3c was observed in trophozoite-parasitized normal and mutant RBC. A very similar pattern was observed previously in trophozoites developing in G6PD-deficient RBC, that were almost indistinguishable from their counterparts grown in normal RBC.
A) HDAS

B) Beta-Thal Trait

C) Alpha-Thal Trait

D) HbH
Figure 1. Parasite invasion and maturation of *P falciparum* (Palo Alto strain, mycoplasma-free) during three cycles of growth in normal, HbAS, beta-thal trait, alpha-thal trait and HbH E. Inoculum was performed mixing separated normal schizonts with normal or mutant E. Parasitemia of inoculum was adjusted to 4%, final hematocrit of inoculum was 0.5%. Each symbol (open, normal E; closed, mutant E) is the mean value±SD (vertical bars) of 5 experiments. Invasion (I): ratio between ring parasitemia measured 14-18 hours after inoculum and inoculum parasitemia (*first cycle invasion*), or ratio between ring parasitemia measured 62-66 hours after inoculum and trophozoite parasitemia measured 40-44 hours after inoculum (*second cycle invasion*) or ratio between ring parasitemia measured 88-92 hours after inoculum and trophozoite parasitemia measured 62-66 hours after inoculum (*third cycle invasion*). Maturation (M): ratio between trophozoite parasitemia measured 34-38 hours after inoculum and ring parasitemia measured 14-18 hours after inoculum (*first cycle maturation*), or ratio between trophozoite parasitemia measured 82-86 hours after inoculum and ring parasitemia measured 62-66 hours after inoculum (*second cycle maturation*), or ratio between trophozoite parasitemia measured 102-106 hours after inoculum and ring parasitemia measured 82-86 hours after inoculum (*third cycle maturation*).

Figure 2. Membrane-bound hemichromes, autologous IgG and complement C3c fragment; aggregated band 3 and phagocytosis in nonparasitized normal and mutant RBC. Parameters were measured in nonparasitized normal control (C), alpha-thal trait (A), beta-thal trait (B), HbAS (AS) and HbH (H) RBC. Hemichromes are expressed in nmol/mL membranes; aggregated band 3 as percentage aggregated band 3 over total band 3; membrane-bound autologous IgG and C3c as milliabsorbance units/min/10⁷ RBC; phagocytosis as number of phagocytosed RBC per monocyte. Data are mean values±SD (vertical bars). Numbers of separate experiments each performed with a different donor were: HbAS, 11; beta-thal trait, 12; alpha-thal trait, 4; HbH, 5. Significance of differences between nonparasitized normal and mutant RBC was assessed by *t*-test for paired samples. *, p<0.001; **, p<0.01; no asterisk, p>0.05.
Figure 3. Membrane-bound hemichromes, autologous IgG and complement C3c fragment; aggregated band 3 and phagocytosis in nonparasitized normal and mutant RBC, and in ring-parasitized normal and mutant RBC. Parameters were measured in nonparasitized normal controls (NP-N), nonparasitized mutant controls (NP-M), normal rings (RP-N) and mutant rings (RP-M) in HbAS, beta-thal trait, alpha-thal trait and HbH RBC. Hemichromes are expressed in nmoles/mL membranes; aggregated band 3 as percentage aggregated band 3 over total band 3; membrane-bound autologous IgG and C3c as milliabsorbance units/min/10^7 RBC; phagocytosis as number of phagocytosed RBC per monocyte. Note that the ordinate values may vary considerably in the different conditions. Data are mean values ± SD (vertical bars). Numbers of separate experiments each performed with a different donor were: HbAS, 11; beta-thal trait, 12; alpha-thal trait, 4; HbH, 5. Significance of differences between normal rings (RP-N) and mutant (RP-M) rings was assessed by t-test for paired samples. *, p<0.001; **, p<0.01; no asterisk, p>0.05.
Figure 4. Effect of blockage of the monocyte complement receptor type 1 (CR1, CD35) by monoclonal antibody J3D3 on the phagocytosis of nonparasitized normal and mutant (beta-thal trait) RBC, ring-parasitized and trophozoite-parasitized normal and mutant RBC. Adherent monocytes (approx. 50,000 monocytes per well) were incubated at 37°C for 30 min with (+) or without (-) 20 µg/mL purified anti-CR1 monoclonal antibody J3D3. After washing of the monocytes, phagocytosis of nonparasitized normal (N, open bars) and mutant (M, black bars) RBC, ring-parasitized and trophozoite-parasitized normal and mutant RBC was performed as detailed in Materials and Methods. Phagocytosis is expressed as number of phagocytosed RBC per monocyte. Data are mean values±SD (vertical bars). Numbers of separate experiments each performed with a different donor were: nonparasitized normal and mutant RBC, 4; ring-parasitized normal RBC, 3; ring-parasitized mutant RBC, 4; trophozoite-parasitized normal RBC, 3; trophozoite-parasitized mutant RBC 4. Significance of differences between treated cells (+) and their corresponding untreated (-) controls was assessed by t-test for paired samples. *, p<0.001; **, p<0.01; no asterisk, p>0.05.
A) HbAS

B) BETA-THAL TRAIT

Figure 5. Membrane-bound hemichromes, autologous IgG and complement C3c fragment; aggregated band 3 and phagocytosis in trophozoite-parasitized normal and mutant RBC. Parameters were measured in trophozoite-parasitized normal RBC (open bars) and trophozoite-parasitized mutant RBC (black bars) in HbAS (panel A) and beta-thal trait (panel B). Hemichromes are expressed in nmoles/mL membranes; aggregated band 3 as percentage aggregated band 3 over total band 3; membrane-bound autologous IgG and C3c as milliabsorbance units/min/10^7 RBC; phagocytosis as number of phagocytosed RBC per monocyte. Data are mean values±SD (vertical bars). Numbers of separate experiments each performed with a different donor were: controls, 8; HbAS, 4; beta-thal trait, 4. Significance of differences between normal and mutant trophozoites was assessed by t-test for paired samples. *, p<0.001; **, p<0.01; no asterisk, p>0.05

Discussion

The mutations considered in this study (HbAS, beta-thal and alpha-thal trait) belong to a group of widespread RBC mutations that confer protection against *P. falciparum* malaria. Protecting mutations also include other hemoglobinopathies (homozygous hemoglobin C, hemoglobin E), a band 3 defect (Southeast Asian Ovalocytosis, SAO) and G6PD-deficiency. Evidence for protection is based on case-control studies on mortality and severity of disease, and on geographic coincidence of distribution of mutants and past or present malaria. The molecular nature of HbAS, beta-thal and alpha-thal trait, and G6PD-deficiency is different. However, in all cases affected RBC show...
increased production of reactive oxygen species (ROS), due to intrinsic characteristics of HbS in HbAS, to unpaired globin chains in the thalassemias and to defective anti-oxidant defence in G6PD deficiency. Summing up observations in normal senescent RBC, pathological or artificially modified RBC, and ring-parasitized RBC, it appears that oxidative events leading to enhanced phagocytosis are in sequence: increased denaturation of Hb, membrane binding of hemichromes and free iron; aggregation of band 3 and deposition of antibodies and complement C3c fragments. Nonoxidative aggregation of band 3 was also found to enhance opsonin deposition and phagocytosis without hemichrome deposition. Aim of this work was to explain why rings developing in beta-thal, sickle-cell trait RBC and HbH-RBC were phagocytosed more intensely than ring-parasitized normal RBC. Based on our observation that oxidative membrane damages, deposition of opsonins and phagocytosis were higher in ring-parasitized mutant RBC compared to “normal” rings we suggest that rings developing in mutant RBC were subjected to a double oxidative stress: a first one exerted by the developing parasite and a second specifically generated by the mutation and additional to the first one. Phagocytosis was similar in trophozoites grown in normal and mutant RBC, as observed previously in trophozoites grown in G6PD-deficient RBC. Damages inflicted by mature parasite forms are very profound and overshadow the baseline differences in normal and mutant RBC. In trophozoites, a larger share of phagocytic recognition does not depend on band 3 aggregation but relies on exposure of other molecules. For example, exposure of PS was remarkable in both mutant and non-mutant trophozoite-parasitized RBC (not shown). Interestingly, hemichromes were significantly increased in trophozoites grown in sickle- and beta thal-trait RBC, whereas aggregated band 3 and phagocytosis did not further increase and were similar to trophozoites grown in normal RBC. Possible reasons may reside in the limited amount of mobile band 3 in the RBC membrane and in the physical limit of 10 RBC ingested per monocyte.

The evident similarities amongst many protective mutations have been noted in several studies (see ref 11 and 38 for review). Not surprisingly, higher levels of bound antibodies and more intense phagocytosis have been described in parasitized thalassemic and other mutant RBC. Thus, enhanced removal of parasitized mutant RBC by the host’s immune system has been suggested as the mechanism underlying resistance. The model presented here is also based on preferential immunological removal of mutant RBC, but it has the distinctive feature that only phagocytosis of ring-parasitized mutant RBC is selectively enhanced, while phagocytosis of trophozoites is very high but quite similar in normal and mutant cells. This model of resistance based on enhanced phagocytosis of ring-parasitized mutant RBC was originally proposed for G6PD-deficiency and is now expanded to include HbAS, beta-thal trait, and HbH. The last condition has no selective value against malaria, since it is accompanied by severe hematologic symptoms. It has been added to show that the different behavior of beta-thal trait (enhanced ring phagocytosis) and alpha-thal trait (no enhancement of ring phagocytosis) resides in the different amount of oxidative damage between the two thalassemic conditions. As soon as the damage increases, as in HbH disease, ring-phagocytosis is enhanced as well.

Supporting evidence that preferential phagocytosis of mutant rings is occurring in vivo can be only provided by mutations with phenotypically distinct mutant and normal RBC populations in the same subject, such as G6PD-deficient female heterozygotes that have a mosaic RBC population, one G6PD-deficient and one normal. Indeed, in malarious G6PD-deficient heterozygous females, a prevalence of parasitized normal RBC vastly in excess over parasitized deficient RBC was described. This unbalance is best explained by the selective removal (“suicidal infection”) of ring-parasitized deficient RBC from circulation. A similar behavior was observed in malarious HbAS
In HbAS, all RBC are genetically equivalent and contain both HbA and HbS; and, a priori, have the same chance of being parasitized and to sickle. However, ring-parasitized HbAS RBC were found to sickle approx. 6 times as readily as nonparasitized cells,\textsuperscript{12,41} generating a double, phenotypically different population: parasitized sickled cells, more prone to be phagocytosed; and nonparasitized non-sickled cells. Indeed, HbAS malarious patients showed a remarkable prevalence of non-sickled vs sickled ring-parasitized RBC in periferal blood,\textsuperscript{42} an unbalanced situation comparable to G6PD-deficient malarious heterozygotes.\textsuperscript{40} Also malarious SAO heterozygotes, a mutation not considered in detail here, have a double population of ovalocytic and normal RBC and displayed selective disappearance of ovalocytic RBC, in accordance with their preferential phagocytosis.\textsuperscript{43} SAO is caused by a band 3 mutation due to a 9-aminoacid deletion in the N-terminal of band 3, producing very rigid RBC with higher amounts of immobile, microaggregated band 3.\textsuperscript{44,45} It is likely that the presence of the parasite may further enhance the increased baseline values of aggregated band 3, reproducing by a partially different mechanism the situation observed here in ring-parasitized sickle-cell and beta-thal trait RBC.

Enhanced phagocytosis of ring-parasitized mutant RBC may be advantageous to the host in several ways. A first advantage is reduction in parasite growth and parasite density, observed in patients with HbAS and beta-thal trait\textsuperscript{46,47} but not in patients with alpha-thal trait. Secondly, phagocytosed ring-forms are digested rapidly by monocytes, and the process repeated without loss of efficiency.\textsuperscript{48} By contrast, more mature forms of the parasite, although actively phagocytosed, severely affect important functions of the monocyte, such as the ability to repeat the phagocytic process.\textsuperscript{48} Adverse effects elicited by ingestion of hemozoin-containing parasite forms include enhanced production of inflammatory cytokines, inability to kill ingested bacteria, to perform repeated cycles of phagocytosis, to express class II and other membrane antigens upon interferon gamma stimulation, and to correctly process antigens.\textsuperscript{48-50} Endothelial functions are also impaired by hemozoin, and adhesion of mature parasite forms to dendritic cells and macrophages down-regulates innate and acquired immune responses.\textsuperscript{50-53} Thirdly, lower numbers of trophozoites and schizonts, that adhere to endothelia in several important organs (lungs, kidneys, brain, bone marrow and placenta) and provoke severe symptoms (for example, cerebral malaria; placental malaria; possibly dyserythropoiesis and respiratory distress) may also lead to less severe disease and lower mortality.\textsuperscript{52,53} Lastly, phagocytosis of ring-parasitized normal and mutant RBC was accompanied by a very modest oxidative burst by monocytes (not shown). Also, complement-mediated phagocytosis was shown to induce a reduced cytokine output by the phagocytic cells.\textsuperscript{54}

Alpha-thal trait rings were not different from non-mutant rings as to hemichrome levels, deposition of removal markers and phagocytosis, and were clearly distinct from beta-thal and HbH rings. All thalassemic syndromes are characterized by unbalanced globin chain synthesis and membrane deposition of excess unpaired alpha- or beta-chains in beta- and alpha-thal trait, respectively. However, pathophysiological consequences are very different. A number of studies (see Schrier et al for review\textsuperscript{55}) and present data indicate that membrane deposition of alpha-chains in beta-thal inflicts distinctly more severe damages, compared to deposition of beta-chains. For example, mechanical stability of membranes was increased in alpha-thal and markedly decreased in beta-thal;\textsuperscript{56} and alpha-chains but not beta-chains bound band 3 cytoplasmic domains with high affinity and positive cooperativity.\textsuperscript{57} The modest degree of alterations observed in alpha-trait nonparasitized and ring-parasitized RBC would exclude the same pattern of resistance to be operating in vivo. There is no doubt that alpha-thal trait is protective, as shown by its coincidental presence with past or present falciparum malaria, as in the Tharu population in Nepal,\textsuperscript{58} in Melanesia and Africa.\textsuperscript{59-62} However, careful studies
by the Oxford group have shown that significantly lower prevalence of severe malaria in thal-t trait children was not accompanied by any difference in parasite density or mortality due to malaria complications. These studies and a study by a different group in Africa found evidence for raised rather than reduced incidence of mild malaria in children carrying alpha-thal. Most likely, enhanced ring-phagocytosis is not operating in alpha-thal trait, whereby the suggestion that alpha-thal protects by predisposing to mild malaria in early life and provoking a cross-vaccination by coincidental co-infection with \textit{P. vivax} may offer an interesting alternative explanation.

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\textbf{References}


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Enhanced phagocytosis of ring-parasitized mutant erythrocytes. A common mechanism that may explain protection against falciparum-malaria in sickle-trait and beta-thalassemia-trait

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