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

Kodjo Ayi, Franco Turrini, Antonio Piga, and Paolo Arese

High frequency of erythrocyte (red blood cell [RBC]) genetic disorders such as sickle cell trait, thalassemia trait, homozygous hemoglobin C (Hb-C), and glucose-6-phosphate dehydrogenase (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 RBCs. We suggested enhanced phagocytosis of rings, the early intraerythrocytic form of the parasite, as an alternative explanation for protection in G6PD deficiency. We show here that *P falciparum* developed similarly in normal RBCs and in sickle trait, beta- and alpha-thalassemia trait, and HbH RBCs. We also show that membrane-bound hemichromes, autologous immunoglobulin G (IgG) and complement C3c fragments, aggregated band 3, and phagocytosis by human monocytes were remarkably higher in rings developing in all mutant RBCs considered except alpha-thalassemia trait. Phagocytosis of ring-parasitized mutant RBCs was predominantly complement mediated and very similar to phagocytosis of senescent or damaged normal RBCs. Trophozoite-parasitized normal and mutant RBCs were phagocytosed similarly in all conditions examined. Enhanced phagocytosis of ring-parasitized mutant RBCs may represent the common mechanism for malaria protection in nonimmune individuals affected by widespread RBC mutations, while individuals with alpha-thalassemia trait are likely protected by a different mechanism. (Blood. 2004;104:3364-3371)

Introduction

High frequency of hemoglobinopathies such as sickle cell trait, thalassemia trait, homozygous hemoglobin C (Hb-C) and Hb-E; 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 Roberts et al[1]; Weatherall[2]; Greene[3]; and Serjeantson et al[4] for reviews). Protection has been attributed in some studies to defective invasion or growth of the parasite in the mutant erythrocytes (red blood cells [RBCs]),[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 mechanisms explaining impaired growth of the parasite in the mutant RBCs.[1-5,11-13] Other studies have found increased deposition of nonspecific autologous antibodies on malaria-parasitized mutant RBCs and suggested phagocytic elimination as the possible mechanism of protection in nonimmune subjects.[14-16] Recently, we found that several strains of *P falciparum* developed similarly in normal and G6PD-deficient RBCs.[9] However, ring-parasitized G6PD-deficient RBCs bound more opsonins such as autologous immunoglobulin G (IgG) and complement C3c fragments, and were phagocytosed 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 RBCs (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 mutants considered except alpha-thal trait. Enhanced phagocytosis of ring-parasitized mutant RBCs was mostly complement mediated and very similar to phagocytic recognition and removal of senescent or oxidatively damaged normal RBCs.[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

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Table 1. Hematologic parameters and genotype in HbAS and beta-thal trait subjects

Subject no.	Sex	Age, y	Hb, g/dL	MCV, fL	MCH, pg	Genotype	HbA2, %	HbF, %
HbAS subjects								
1	F	27	13.2	81.3	26.6	Codon 6 (A>T)/N	2.5	1.2
2	F	22	14.0	86.1	30.2	Codon 6 (A>T)/N	2.4	0.5
3	M	28	15.2	91.5	31.4	Codon 6 (A>T)/N	2.9	1.0
4	F	20	12.7	77.1	28.9	Codon 6 (A>T)/N	2.3	1.1
5	M	34	14.4	82.4	29.1	Codon 6 (A>T)/N	2.5	1.1
6	F	29	13.3	76.3	26.2	Codon 6 (A>T)/N	1.9	1.6
7	F	25	14.1	84.8	28.8	Codon 6 (A>T)/N	2.4	0.7
8	M	27	14.9	80.9	30.1	Codon 6 (A>T)/N	2.8	0.6
9	M	31	13.6	83.4	27.7	Codon 6 (A>T)/N	2.6	1.1
10	F	31	13.1	78.5	26.6	Codon 6 (A>T)/N	2.4	1.2
11	F	28	12.6	81.0	25.5	Codon 6 (A>T)/N	2.2	1.3
Beta thal trait subjects								
1	F	23	10.6	63.6	18.5	Codon 39 (C>T)/N	4.6	0.7
2	M	28	12.4	68.5	20.3	Codon 39 (C>T)/N	4.7	0.0
3	F	40	10.6	63.8	18.8	Codon 39 (C>T)/N	5.4	2.3
4	M	39	12.5	63.0	21.0	Codon 39 (C>T)/N	5.0	2.6
5	F	41	11.4	61.0	20.3	Codon 39 (C>T)/N	5.8	0.5
6	M	48	12.1	64.0	18.6	Codon 39 (C>T)/N	5.7	0.0
7	M	51	14.3	62.5	20.8	Codon 39 (C>T)/N	6.0	0.6
8	F	58	10.6	69.0	20.7	Codon 39 (C>T)/N	5.5	2.7
9	F	35	12.3	67.0	21.2	Codon 39 (C>T)/N	4.9	3.0
10	F	51	12.9	73.0	21.4	Codon 39 (C>T)/N	5.1	1.2
11	F	35	10.4	59.8	18.9	Codon 39 (C>T)/N	6.0	1.1
12	F	31	11.1	61.7	19.6	Codon 39 (C>T)/N	5.6	0.9

MCV indicates mean corpuscular volume; MCH, mean corpuscular hemoglobin.

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, sodium dodecyl sulfate (SDS), bovine serum albumin, and Tween-20 were from Sigma (St Louis, MO). Octaethylene glycol mono-n-dodecyl ether (C12E8) was from Nikkei Chemical (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 (Dudingen, Switzerland). All other reagents were purchased from common commercial sources.

Preparation of RBCs from healthy and mutant subjects, cultivation of *P falciparum,* and stage-separation of parasitized RBCs

Control RBCs were obtained from hematologically healthy subjects aged 20 to 55 years. Controls were staff members or blood donors from the local blood bank. All controls had normal hematologic parameters and normal G6PD activity. The following mutant RBCs were obtained from healthy subjects of both sexes aged 20 to 58 years: heterozygous beta-thalassemia (Codon beta-39 (C>T)/N), beta-thal trait; alpha-thalassemia (homozygotes for alpha plus-thalassemia, -α3.7/-α3.7, alpha-thal trait; compound heterozy-gotes 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 Tables 1-2. 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 polymerase chain reaction methodology.[20,21] None of the control or mutant subjects was G6PD deficient. Blood from healthy and mutant individuals, anticoagulated with citrate-phosphate-dextrose with adenine, was used immediately after withdrawal. RBCs 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 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 20 mM 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, 177.6, and FCR-3. All strains were *mycoplasma*-free. Parasites were cultivated at 1% hematocrit, synchronized, and separated as described.[22,23] Culture medium (RPMI 1640 medium with Hepes and glutamine) was supplemented with 10% fresh autologous serum, 20 mM glucose, and 0.8 mM adenine (final

Table 2. Hematologic parameters and genotype in alpha-thal trait and HbH subjects

Subject no.	Sex	Age, y	Hb, g/dL	MCV, fL	MCH, pg	Genotype	HbA2, %	HbF, %	HbH, %
1	M	58	14.4	81.0	28.5	$-\alpha^{3.7}/-\alpha^{3.7}$	2.1	0.5	0
2	F	55	12.4	67.0	20.7	$-\alpha^{3.7}/-\alpha^{3.7}$	2.3	0.5	0
3	F	35	12.8	68.0	21.2	$-\alpha^{3.7}/-\alpha^{3.7}$	1.0	0.9	0
4	M	23	12.6	73.0	21.4	$-\alpha^{3.7}/-\alpha^{3.7}$	1.1	0.7	0
5	F	30	8.5	69.4	17.1	--/-α(HbH)	2.0	0.5	12
6	F	26	8.9	71.5	19.1	--/-α(HbH)	1.4	1.1	12
7	M	24	8.4	55.5	15.6	--/-α(HbH)	1.3	1.1	5
8	M	23	9.9	59.4	17.1	--/-α(HbH)	1.7	0.3	7
9	F	25	8.9	65.1	19.3	--/-α(HbH)	1.0	0.9	10

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 RBCs. Indeed, the standard culture conditions at high CO_2 concentration and slightly lower pH values in RBCs had been previously shown to induce changes in healthy controls that led to increased phagocytic rate.[24] For all studies, parasitemia of inoculum was adjusted to 20% schizont-parasitized RBCs (schizonts) for isolation of rings and to 5% schizonts for isolation of trophozoites and schizonts. At 14 to 18 hours after inoculum (rings), 34 to 38 hours (trophozoites), and 40 to 44 hours after inoculum (schizonts), rings, trophozoites, and schizonts were separated on Percoll-mannitol gradients.[22,23] The parasitemia was usually 35% to 45% rings, more than 95% trophozoites, and more than 95% schizonts. Except for the invasion and maturation experiments, parasitized RBCs were isolated during the first cycle of parasite growth. The total number of nonparasitized and parasitized RBCs was counted electronically by Coulter Counter (Coulter, Birmingham, United Kingdom). To assess total parasitemia and relative contribution of rings, trophozoites, and schizonts, slides were prepared from cultures at indicated times and stained with Diff-Quik parasite stain, and 400 to 1000 cells were examined microscopically.

Assessment of parasite invasion and maturation

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

Opsonization of RBCs

Freshly separated nonparasitized and parasitized RBCs were washed 3 times in wash medium (130 mM NaCl,10 mM Hepes, 10 mM glucose, pH 7.4). Washed RBCs 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 RBCs were washed 3 times in wash medium. RBC-bound IgGs were measured after labeling RBCs with anti–human IgG developed in goat for one hour 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% to 25% rings were extrapolated to 100% rings parasitemia using the following calculation: $I = (TOT - N \times n)/(1 - n)$, where I indicates the 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; and n, fraction of nonparasitized RBCs.[9,23]

Preparation and extraction of hypotonic membranes with nonionic detergent C12E8

Standard hypotonic membranes from nonparasitized and stage-separated parasitized RBCs were prepared at 0°C by hemolysis in hemolysis buffer (5 mM sodium phosphate, 1 mM EDTA, pH 8.0) and 2 washes. Freshly prepared membranes (0.5 mL) were mixed as indicated[23] with 1 mL extraction buffer (130 mM NaCl, 10 mM Hepes, 10 mM NEM, 1 mM EDTA, 1 mM PMSF, 0.05 U/mL aprotinin, 0.5 μg/mL leupeptin, 20 μg/mL pepstatin, and 1.5% [vol/vol] nonionic detergent C12E8, pH 7.4) at 37°C for 20 minutes under moderate shaking and then pelletted for 15 minutes at 13 000 rpm in a refrigerated Eppendorf microfuge. The clear supernatant (C12E8 extract) was immediately separated from the pellet and used for gel-filtration chromatography.

Gel-filtration chromatography of the C12E8 extract

The C12E8 extract (1 mL) was loaded onto a Sepharose CL-6B column (excluded volume > 4000 kDa molecular weight) and separated at a flow rate of 0.9 mL/min. The effluent was collected in 0.8-mL fractions (C12E8 fractions) as indicated.[23]

Assay of membrane-bound hemichromes

Membrane-bound hemichromes were assayed in standard hypotonic membranes prepared as indicated (see "Preparation and extraction of hypotonic membranes with nonionic detergent C12E8) by measuring absorbance at 560, 577, and 630 nm, using millimolar absorptivity values of 8.6, 6.8, and 0.92, respectively.[25] Due to the great variability in protein content in RBC membrane preparations, membrane volume was selected as a reference and hemichromes were expressed as nmol hemichrome/mL membrane.[26] Membrane volume showed low intersubject variability and roughly corresponded to the original RBC volume in both normal and mutant RBCs.

Assay of aggregated band 3

Aggregated band 3 was assayed in the C12E8 fractions of nonparasitized and stage-separated parasitized RBCs previously labeled by the band 3–specific fluorescent label eosin-5-maleimide as described.[23] In order to quantitate the percentage of aggregated band 3, eosin-5-maleimide–labeled band 3 was assayed by fluorometry in all C12E8 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 RBCs per monocyte as indicated.[27]

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 Micheal D. Kazatchkine, Paris, France. Adherent monocytes (approximately 50 000 monocytes per well) were incubated at 37°C for 30 minutes in 1 mL 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.[24]

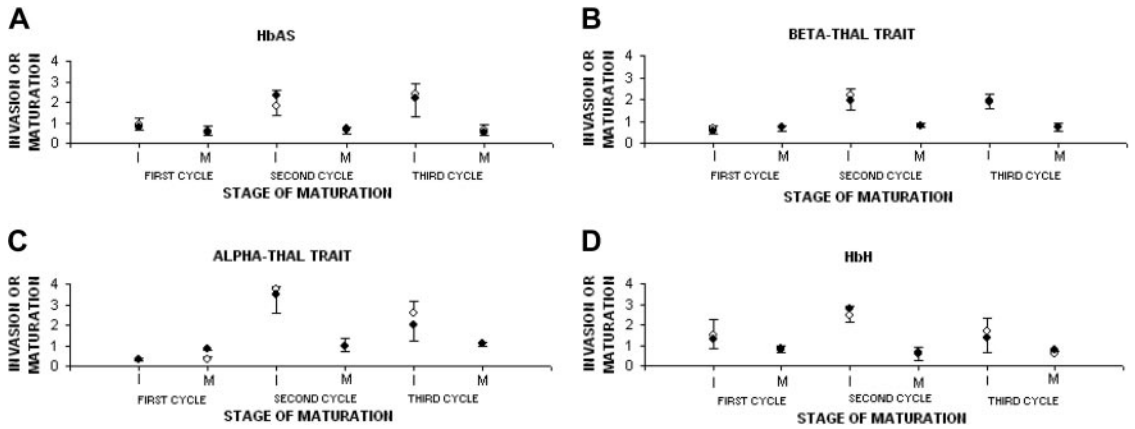

Figure 1. Parasite invasion and maturation of *P falciparum* (Palo Alto strain, *mycoplasma*-free) during 3 cycles of growth in normal, HbAS, beta-thal trait, alpha-thal trait, and HbH E. Inoculum was performed mixing separated normal schizonts with normal or mutant E. Parasitemia of inoculum was adjusted to 4%, final hematocrit of inoculum was 0.5%. Each symbol (○, normal E; ●, mutant E) is the mean value ± SD (vertical bars) of 5 experiments. Invasion (I): ratio between ring parasitemia measured 14 to 18 hours after inoculum and inoculum parasitemia (first cycle invasion); or ratio between ring parasitemia measured 62 to 66 hours after inoculum and trophozoite parasitemia measured 40 to 44 hours after inoculum (second cycle invasion); or ratio between ring parasitemia measured 88 to 92 hours after inoculum and trophozoite parasitemia measured 62 to 66 hours after inoculum (third cycle invasion). Maturation (M): ratio between trophozoite parasitemia measured 34 to 38 hours after inoculum and ring parasitemia measured 14 to 18 hours after inoculum (first cycle maturation); or ratio between trophozoite parasitemia measured 82 to 86 hours after inoculum and ring parasitemia measured 62 to 66 hours after inoculum (second cycle maturation); or ratio between trophozoite parasitemia measured 102 to 106 hours after inoculum and ring parasitemia measured 82 to 86 hours after inoculum (third cycle maturation).

Statistical analysis

Significance of differences was assessed by *t* test for paired samples.

Results

Hematologic parameters and genetic characteristics of mutant RBCs

The hematologic and genetic characteristics of mutant RBC donors are presented in Tables 1-2. 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 RBCs

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

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

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 RBCs kept for comparable time periods under the same culture conditions as parasitized RBCs ("Materials and methods"). The highest level of damage was in nonparasitized HbAS and HbH RBCs, while alpha-thal trait RBCs 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 RBCs compared with matched ring-parasitized normal RBCs. With the exception of alpha-thal trait RBCs, all ring-parasitized mutant RBCs had distinctly higher levels of all considered parameters. Phagocytosis was also very remarkably increased in ring-parasitized RBCs in all mutations except alpha-thal trait. As expected from the baseline situation, rings developing in HbH RBCs 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 kDa molecular weight), indicating heme binding

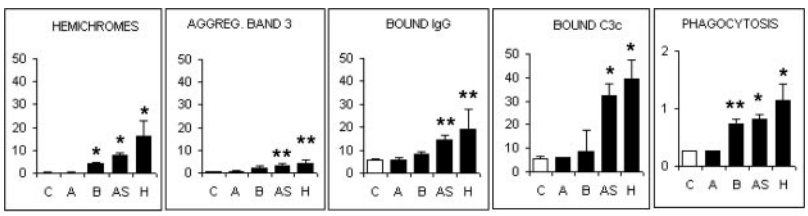

Figure 2. Membrane-bound hemichromes, autologous IgG, and complement C3c fragment; aggregated band 3 and phagocytosis in nonparasitized normal and mutant RBCs. Parameters were measured in nonparasitized normal control (C), alpha-thal trait (A), beta-thal trait (B), HbAS (AS), and HbH (H) RBCs. Hemichromes 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 RBCs; and phagocytosis, as number of phagocytosed RBCs per monocyte. Data are mean values ± SD (vertical bars). Numbers of separate experiments each performed with a different donor were as follows: HbAS, 11; beta-thal trait, 12; alpha-thal trait, 4; and HbH, 5. Significance of differences between nonparasitized normal and mutant RBCs was assessed by *t* test for paired samples. *$P < .001$; **$P < .01$; and no asterisk, $P > .05$.

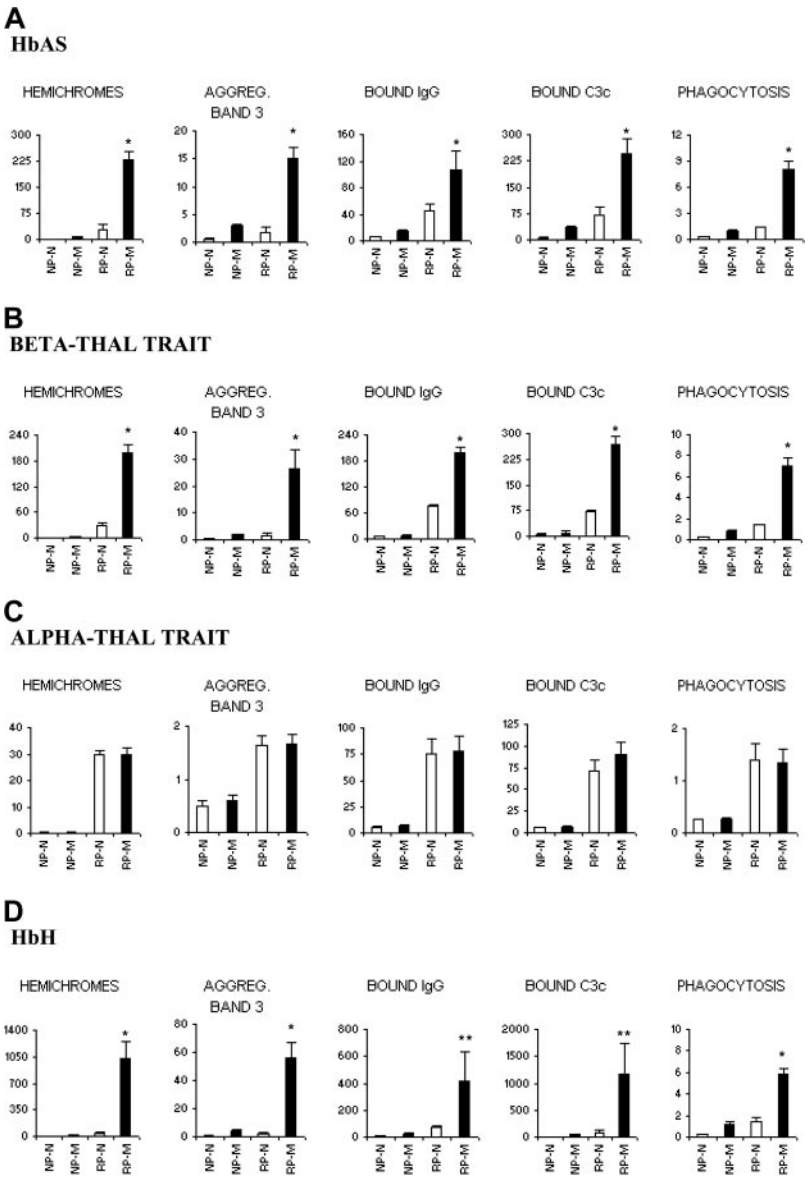

Figure 3. Membrane-bound hemichromes, autologous IgG, and complement C3c fragment; aggregated band 3 and phagocytosis in nonparasitized normal and mutant RBCs, and in ring-parasitized normal and mutant RBCs. Parameters were measured in nonparasitized normal controls (NP-N), nonparasitized mutant controls (NP-M), normal rings (RP-N), and mutant rings (RP-M) in HbAS, beta-thal trait, alpha-thal trait, and HbH RBCs. 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 RBCs; and phagocytosis, as number of phagocytosed RBCs 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 as follows: HbAS, 11; beta-thal trait, 12; alpha-thal trait, 4; and HbH, 5. Significance of differences between normal rings (RP-N) and mutant (RP-M) rings was assessed by *t* test for paired samples. $^{*}P < .001$; $^{**}P < .01$; and no asterisk, $P > .05$.

to aggregated, high-molecular-weight membrane components (not shown). The absorption spectrum of the heme-containing fractions corresponded to that of hemichromes.[25] The same fractions contained aggregated band 3, which was localized by labeling hypotonic membranes of ring-parasitized RBCs with specific fluorescent band 3 label eosin-5-maleimide (not shown). This chromatographic behavior showing coelution 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.[27] The role of complement as opsonin was tested in ring-parasitized beta-thal trait RBCs. 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 RBCs by approximately 80% and 94%, respectively, in agreement with previous observations.[23,24] Decrease of C3-mediated phagocytosis was less pronounced in trophozoites (46% and 43% reduction in trophozoite-parasitized beta-thal trait and normal RBCs, respectively), indicating that IgG and scavenger receptors[28] may play a more significant role in phagocytosis of mature parasite forms. Latter data are in

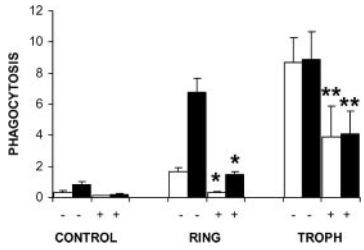

Figure 4. Effect of blockage of the monocyte complement receptor type 1 (CR1, CD35) by monoclonal antibody J3D3 on the phagocytosis of nonparasitized normal and mutant (beta-thal trait) RBCs, and ring-parasitized and trophozoite-parasitized normal and mutant RBCs. Adherent monocytes (approximately 50 000 monocytes per well) were incubated at 37°C for 30 minutes with (+) or without (−) 20 μg/mL purified anti-CR1 monoclonal antibody J3D3. After washing of the monocytes, phagocytosis of nonparasitized normal (□) and mutant (■) RBCs, ring-parasitized and trophozoite-parasitized normal and mutant RBCs was performed as detailed in "Materials and methods." Phagocytosis is expressed as number of phagocytosed RBCs per monocyte. Data are mean values ± SD (vertical bars). Numbers of separate experiments each performed with a different donor were as follows: nonparasitized normal and mutant RBCs, 4; ring-parasitized normal RBCs, 3; ring-parasitized mutant RBCs, 4; trophozoite-parasitized normal RBCs, 3; and trophozoite-parasitized mutant RBCs, 4. Significance of differences between treated cells (+) and their corresponding untreated (−) controls was assessed by *t* test for paired samples. $^{*}P < .001$; $^{**}P < .01$; and no asterisk, $P > .05$.

agreement with previous observations in trophozoite-parasitized G6PD-deficient RBCs.[23,24] 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 RBCs cultivated in the presence of 100 μM beta-mercaptoethanol, confirming previous data obtained with nonmutant parasitized RBCs (not shown).[23]

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

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

As shown in Figure 5, the differences between ring-parasitized normal and mutant RBCs largely vanished at later parasite development stages. Trophozoites developing in normal or mutant (HbAS and beta-thal trait) RBCs were phagocytosed to the physical limit of the monocyte (approximately 10 parasitized RBCs per monocyte),[27] and differences between parasitized normal and mutant RBCs appeared to vanish. Accordingly, the same generation and deposition of aggregated band 3, IgG, and C3c was observed in trophozoite-parasitized normal and mutant RBCs. A very similar pattern was observed previously in trophozoites developing in G6PD-deficient RBCs, which were almost indistinguishable from their counterparts grown in normal RBCs.[9]

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.[1,2] Protective mutations also include other hemoglobinopathies (homozygous hemoglobin C, hemoglobin E),[29,30] a band 3 defect (Southeast Asian ovalocytosis, SAO),[4] and G6PD deficiency.[3] 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 RBCs 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 antioxidant defense in G6PD deficiency.[31-33] Summing up observations in normal senescent RBCs, pathologic or artificially modified RBCs, and ring-parasitized RBCs, 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.[17,18,23,33-35] Nonoxidative aggregation of band 3 was also found to enhance opsonin deposition and phagocytosis without hemichrome deposition.[36] The aim of this work was to explain why rings developing in beta-thal, sickle cell trait RBCs, and HbH RBCs were phagocytosed more intensely than ring-parasitized normal RBCs. Based on our observation that oxidative membrane damages as well as deposition of opsonins and phagocytosis were higher in ring-parasitized mutant RBCs compared with "normal" rings we suggest that rings developing in mutant RBCs 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 RBCs, as observed previously in trophozoites grown in G6PD-deficient RBCs.[9] Damages inflicted by mature parasite forms are very profound and overshadow the baseline differences in normal and mutant RBCs. In trophozoites, a larger share of phagocytic recognition does not depend on band 3 aggregation but relies on exposure of other molecules.[24] For example, exposure of PS was remarkable in both mutant and nonmutant trophozoite-parasitized RBCs (not shown). Interestingly, hemichromes were significantly increased in trophozoites grown in sickle and beta-thal trait RBCs, whereas aggregated band 3 and phagocytosis did not further increase and were similar to trophozoites grown in normal RBCs. Possible reasons may reside in the limited amount of mobile band 3 in the RBC membrane[37] and in the physical limit of 10 RBCs ingested per monocyte.[27]

The evident similarities among many protective mutations have been noted in several studies (see Destro-Bisol et al[11] and Anastasi[38] for review). Not surprisingly, higher levels of bound antibodies and more intense phagocytosis have been described in parasitized thalassemic and other mutant RBCs.[15,16] Thus, enhanced removal of parasitized mutant RBCs by the host's immune system has been suggested as the mechanism underlying resistance.[11,15,16] The model presented here is also based on preferential immunologic removal of mutant RBCs, but it has the distinctive feature that only phagocytosis of ring-parasitized mutant RBCs 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

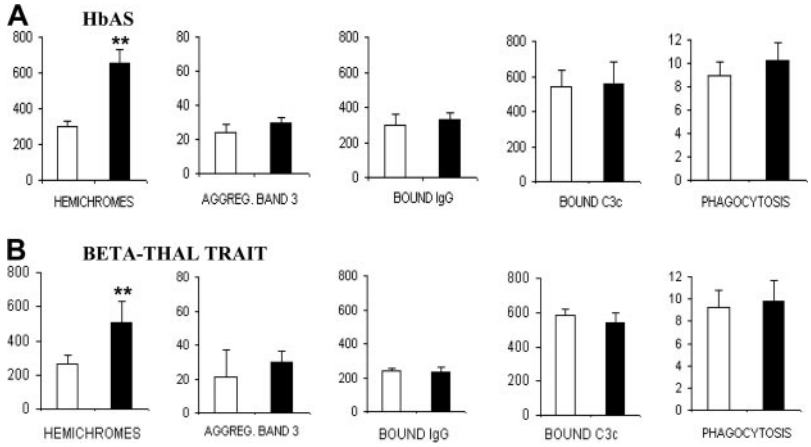

Figure 5. Membrane-bound hemichromes, autologous IgG, and complement C3c fragment; aggregated band 3 and phagocytosis in trophozoite-parasitized normal and mutant RBCs. Parameters were measured in trophozoite-parasitized normal RBCs (□) and trophozoite-parasitized mutant RBCs (■) in HbAS (A) and beta-thal trait (B). 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 RBCs; and phagocytosis, as number of phagocytosed RBCs per monocyte. Data are mean values ± SD (vertical bars). Numbers of separate experiments each performed with a different donor were as follows: controls, 8; HbAS, 4; and beta-thal trait, 4. Significance of differences between normal and mutant trophozoites was assessed by *t* test for paired samples. $^{**}P < .01$; and no asterisk, $P > .05$.

mutant RBCs 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 2 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 provided only 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.[39] Indeed, in malarious G6PD-deficient heterozygous females, a prevalence of parasitized normal RBCs vastly in excess over parasitized deficient RBCs was described.[40] This unbalance is best explained by the selective removal ("suicidal infection") of ring-parasitized deficient RBCs from circulation.[40] A similar behavior was observed in malarious HbAS subjects. In HbAS, all RBCs 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 RBCs were found to sickle approximately 6 times as readily as nonparasitized cells,[12,41] generating a double, phenotypically different population: parasitized sickled cells, more prone to be phagocytosed, and nonparasitized nonsickled cells. Indeed, HbAS malarious patients showed a remarkable prevalence of nonsickled versus sickled ring-parasitized RBCs in peripheral blood,[42] an unbalanced situation comparable with G6PD-deficient malarious heterozygotes.[40] Also malarious SAO heterozygotes, a mutation not considered in detail here, have a double population of ovalocytic and normal RBCs and displayed selective disappearance of ovalocytic RBCs, in accordance with their preferential phagocytosis.[43] SAO is caused by a band 3 mutation due to a 9–amino acid deletion in the N-terminal of band 3, producing very rigid RBCs with higher amounts of immobile, microaggregated band 3.[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 RBCs.

Enhanced phagocytosis of ring-parasitized mutant RBCs 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[46,47] but not in patients with alpha-thal trait. Secondly, phagocytosed ring forms are digested rapidly by monocytes, and the process is repeated without loss of efficiency.[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.[48] Adverse effects elicited by ingestion of hemozoin-containing parasite forms include enhanced production of inflammatory cytokines, and 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.[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.[50-53] Thirdly, lower numbers of trophozoites and schizonts, which 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.[52,53] Lastly, phagocytosis of ring-parasitized normal and mutant RBCs 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.[54]

Alpha-thal trait rings were not different from nonmutant 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, pathophysiologic consequences are very different. A number of studies (see Schrier et al[55] for review) and present data indicate that membrane deposition of alpha-chains in beta-thal inflicts distinctly more severe damages, compared with deposition of beta-chains. For example, mechanical stability of membranes was increased in alpha-thal and markedly decreased in beta-thal[56]; and alpha-chains but not beta-chains bound band 3 cytoplasmic domains with high affinity and positive cooperativity.[57] The modest degree of alterations observed in alpha-trait nonparasitized and ring-parasitized RBCs 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,[58] and in Melanesia and Africa.[59-62] However, careful studies by the Oxford group have shown that significantly lower prevalence of severe malaria in thal-trait children was not accompanied by any difference in parasite density or mortality due to malaria complications.[59,61] These studies and a study by a different group in Africa[62] 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 coinfection with *Plasmodium vivax* may offer an interesting alternative explanation.[61]

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