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## Acute haemolysis in childhood falciparum malaria

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### Abstract

Acute haemolysis associated with clinical episodes of high-level *Plasmodium falciparum* parasitaemia was studied in 20 children from an holoendemic area (coastal Tanzania). The change in blood haemoglobin (Hb) concentration ranged from –46 to +5 g/L during the 72-h observation period and was linearly related to maximum parasitaemia. Balance studies between loss of blood Hb, increase in plasma Hb and appearance of Hb in the urine indicated that extravascular clearance of red cells was the predominant mode of erythrocyte clearance. Most subjects, however, showed minor signs of intravascular haemolysis. The plasma Hb was  $\ll$ 1% of blood Hb and haemoglobinuria was detected in 14/20 children but the excretion of Hb in urine was  $<$ 0.5% of total Hb loss. Haemoglobinuria was, however, a marker of severe haemolysis, since the maximum blood Hb loss in children without haemoglobinuria was 10 g/L. Erythrocyte-bound opsonins known to induce erythrophagocytosis, i.e., complement C3c fragments and autologous IgG, were increased in all patients. In the patients with major haemolysis, the changes correlated to the haemolysis over time. Hence, a similar mechanism for predominantly extravascular erythrocyte clearance may be operative in acute malarial anaemia, normal erythrocyte senescence and other forms of acute haemolysis.

**Keywords:** malaria, *Plasmodium falciparum*, haemolytic anaemia, haemoglobinuria, children, Tanzania

### Introduction

Childhood anaemia due to malaria represents a major medical problem in sub-Saharan Africa but its pathogenesis is complex (ABDALLA *et al.*, 1980; NEWTON *et al.*, 1997). A chronic type of anaemia which is frequently unrelated to current parasitaemia is common in semi-immune individuals living in holoendemic areas (PHILLIPS & PASVOL, 1992). Acute anaemia, superimposed on a low haemoglobin (Hb) level, is an important feature in young African children (EKVALL *et al.*, 1998). Malaria triggers an imbalance in red blood cell (RBC) destruction and production which subsequently leads to anaemia, but there is still no comprehensive understanding of the relative contributions made by intravascular haemolysis, extravascular clearance of infected and uninfected RBC and bone-marrow dysfunction. Intravascular destruction of infected RBCs occurs at merogony (DEVAKUL *et al.*, 1969), and extravascular phagocytosis of infected RBCs is caused by macrophages in the spleen, liver and bone-marrow (AIKAWA *et al.*, 1980; SEED & KREIER, 1980). Uninfected RBCs have a shortened life-span in malaria (PHILLIPS *et al.*, 1986; LOOAREESUWAN *et al.*, 1987) and, irrespective of how they are selected for removal, it has been generally acknowledged that their elimination occurs by erythrophagocytosis (CLARK & CHAUDHRI, 1988). The acute haemolytic anaemia of malaria has been compared with premature RBC ageing (CONRAD, 1969), and a common model unifying the mechanism of RBC removal in normal senescence and in some pathological conditions with acute anaemia has been suggested (ARESE *et al.*, 1991).

The objectives of the present study were to assess quantitatively the predominant mode (extravascular vs intravascular) of haemolysis in acute episodes of childhood falciparum malaria, and to determine the relationship between parasitaemia and loss of blood Hb. The deposition of complement factors and IgG was also studied, including their potential role in the extravascular removal of RBCs.

### Materials and Methods

#### Study subjects

All children aged 6 months to 4 years presenting with a history of fever to the outpatient department of

Kisarawe district hospital, coastal Tanzania, between June and August 1997 were selected as possible study subjects. Out of 92 children with a confirmed axillary temperature  $>$ 38°C, 20 satisfied the inclusion criteria of *Plasmodium falciparum* parasitaemia  $\geq$ 1% and Hb  $>$  50 g/L without signs of cardiac failure. A physical examination including spleen size was made. No child had to be excluded owing to malnutrition. Oral informed consent was obtained from the mother for in-hospital medical treatment and blood collection over 3 days.

#### Clinical follow-up

Approximately 0.75 mL venous blood was collected on admission and then once daily for 3 days under atraumatic conditions. Self-adhesive plastic bags (Coloplast<sup>®</sup>) were attached to small children not in control of urine output for continuous urine collection; glass containers for micturition were used for older children. All specimens were microscopy checked to exclude urinary-tract infection and haematuria of other origin. Twenty-four hour portions of urine were frozen to –20°C for later analysis of Hb content, as described below. Bodyweight, serum sodium and creatinine were determined on admission and on discharge to assess any changes in fluid balance. The children were given effective antimalarial therapy, either quinine alone or sulfadoxine–pyrimethamine and chloroquine. Blood transfusion was considered in any child who developed Hb  $<$  50 g/L together with clinical signs of respiratory distress.

#### Field laboratory methods

Thick and thin blood films were treated with 5% Giemsa stain and *P. falciparum* parasitaemia was estimated against 3000 RBCs. Blood Hb concentration was recorded in duplicate from venous blood, using the portable Haemocue<sup>®</sup> system and quality control kits (Haemocue AB, Sweden). After centrifugation, plasma or serum was atraumatically removed and frozen. Hypotonic RBC membranes were prepared from each blood sample as described by DODGE *et al.* (1963). The membranes were prepared immediately after blood collection and frozen to –20°C until analysis. Part of the RBC membrane collection was accidentally thawed owing to electrical power failure. The thawed samples were however discarded and 62 of the 70 specimens were analysed.

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### Central laboratory methods

Plasma and urine Hb concentrations were analysed by spectrophotometry at the Department of Clinical Chemistry, Huddinge University Hospital, Sweden. Plasma haptoglobin and haemopexin, and serum sodium and creatinine, were analysed by routine methods at Sahlgrenska University Hospital, Sweden. Hb electrophoresis to identify HbS and screening for glucose 6-phosphate dehydrogenase (G6PD)-deficiency were also performed (BEUTLER, 1975).

RBC-bound IgG and complement fragment C3c were measured on the hypotonic membranes prepared from the patients and from apparently healthy black children of comparable age without malaria taken as controls. In brief, membranes were labelled with goat anti-human C3c or rabbit anti-human IgG and mouse second antibodies conjugated to alkaline phosphatase, as described (TURRINI *et al.*, 1994). The alkaline phosphatase activity was measured by visible spectrophotometry at 405 nm using nitroblue tetrazolium/bromochlorophosphate as phosphatase substrate, and expressed as increments of mOD/10  $\mu$ L membrane volume/min. For comparison, 100 mOD/10  $\mu$ L membrane volume/min corresponds to about 225 IgG and 3800 C3c molecules bound per RBC, respectively (TURRINI *et al.*, 1991).

RBC-bound IgG molecules were eluted from the membranes and their specificity determined, as described by TURRINI *et al.* (1994). In brief, aggregation of band 3 can be elicited in normal RBCs by treatment with zinc/BS3 (*bis*-sulphosuccinimidyl-suberate). Eluted autologous IgG from the malaria patients was blotted on RBC membrane proteins prepared from zinc/BS3-treated RBC. After washing, anti-human IgG conjugated to alkaline phosphatase was used for visualizing eluted autologous IgG, and immunoblots were developed with nitroblue tetrazolium/bromochlorophosphate.

### Specific materials

Sigma provided rabbit anti-human IgG antibodies, goat anti-human C3c antibodies, mouse anti-goat and anti-rabbit IgG antibodies conjugated to alkaline phosphatase (all affinity-purified polyclonals), detergents, bovine serum albumin and other chemicals. Electrophoresis material was from Biorad and protein A beads from Pharmacia Biotech; BS3 was from Pierce. Antibodies to band 3 cytoplasmic domain were a gift from P. S. Low (Purdue University, West Lafayette, IN, USA).

### Statistical methods

The Wilcoxon rank-sum test was used for non-normal or sparse continuous data. Paired or 2-sample *t*-tests were used for continuous data with a normal distribution; parasitaemia and plasma Hb were normalized by logarithmic transformation.

### Ethical considerations

Research approvals were obtained from the ethical committees of the Karolinska Institutet in Stockholm (#98175) and the Muhimbili College of Health Sciences in Dar es Salaam. Parents of study subjects provided oral informed consent.

## Results

### Patient data

Twenty children satisfied the study admission criteria of axillary temperature  $>38^{\circ}\text{C}$ , *P. falciparum* parasitaemia  $\geq 1\%$  and admission blood Hb  $> 50$  g/L. The individual characteristics of the study subjects are shown in the Table. The mean age was 20 months (range 6–45) and there was no difference between males and females. One subject was G6PD-deficient (ID #12) and one was identified as HbAS (ID #11); the remaining children were HbAA.

No child received blood transfusion during the study period and only ID #5 required intravenous fluid replacement on a single occasion. Bodyweight, serum sodium and serum creatinine, all markers of fluid balance, were not significantly altered on discharge compared with admission values. Patient ID #5 was transfused after the end of the study.

The geometric mean of individual maximum parasitaemias was 3.6% (range 1.7–20). Individual parasitaemias peaked sometimes on admission ( $n = 11$ ) and sometimes after 24 h ( $n = 8$ ). Only 2 of the 20 subjects were microscopy clear of parasites on the last blood collection.

### Blood haemoglobin concentrations

The median change in blood Hb concentration over the 72-h observation period was  $-13$  g/L (range,  $-46$  to  $+5$ ). Assuming a blood volume of 0.075 L/kg bodyweight (KING, 1998), the median amount of Hb lost from blood during the 72-h observation period was 11 g (maximum 37 g). Major haemolytic episodes, defined as a decrease in blood Hb concentration  $\geq 10$  g/L in 72 h, were observed in 12 subjects; the remaining 8 children showed only temporary or minor changes in Hb. In the 12 patients with major haemolysis, the drop in blood Hb was  $>10\%$  of the admission level. The loss of Hb was positively associated with age ( $P = 0.13$ ). However, after adjusting for admission Hb concentration in a regression model, age was not significant in explaining blood Hb loss (Table). There was no association between spleen size or antimalarial treatment and blood Hb loss.

Percentage parasitaemia on admission was related to blood Hb loss during the 72-h observation period. The best fit for regression of parasitaemia on the reduction in Hb was however obtained by using the individual maximum parasitaemia ( $R^2 = 0.58$ ,  $P < 0.0001$  vs  $R^2 = 0.39$ ,  $P = 0.004$ ) (Fig. 1). Overall, the median blood Hb loss (expressed as the percentage of admission Hb) was 3.7 times (range, 0.5–9) larger than the corresponding reduction in infected cells. Individual differences in the relationship between parasitaemia and blood Hb loss were observed. For example, ID #8 and #15 with analogous Hb on admission and a reduction in parasitaemia of 4% under treatment showed individual blood Hb losses of 28 g/L and 4 g/L, respectively (Table).

### Plasma concentrations of Hb, haptoglobin and haemopexin

Haemoglobinaemia occurred in 13 of the 20 children with detectable plasma Hb concentrations (Table). In these children the geometric mean plasma Hb concentration was 0.25 g/L (range, 0–0.88). At any point in time, the plasma Hb concentrations were  $\ll 1\%$  of

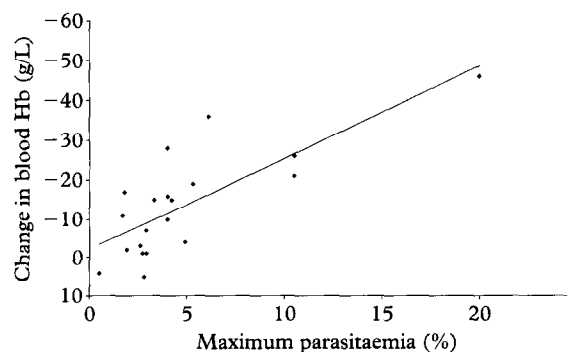


Fig. 1. The relationship between individual maximum parasitaemias and change in blood haemoglobin (Hb) concentrations (g/L) over 72 h in 20 children with haemolysis due to acute *P. falciparum* malaria. Correlation coefficient,  $r = 0.76$ ;  $P < 0.001$ .

Table. Characteristics of 20 children with acute episodes of high-density *P. falciparum* malaria followed for 72 h; Table is arranged in descending order of blood haemoglobin (Hb) loss

ID	Age (months)	Maximum parasitaemia (%)	Admission blood Hb (g/L)	Change in blood Hb (g/L)	Plasma Hb (g/L), median (range)	Urine Hb (g/L), median (range)	Total haemoglobinuria (g Hb/72 h)	Plasma haptoglobin <sup>a</sup> (g/L)	Plasma haemopexin <sup>a</sup> (g/L)
5	27	20.0	89	-46	0.1 (0-0.2)	0.1 (0-0.1)	0.05	0.3	0.2
2	42	6.1	108	-36	0.1 (0-0.2)	0.7 (0.1-0.9)	0.19	0.1	0.7
8	11	4.0	76	-28	0.2 (0.1-0.3)	0.06 (0-0.2)	0.03	0.3	0.5
1	45	10.5	101	-26	0.5 (0.4-0.6)	0.6 (0.4-1)	0.24	0.2	0.5
7	8	10.5	58	-21	0.2 (0-0.3)	0.2 (0-0.4)	0.11	0.3	0.6
19	13	5.3	77	-19	0	0.09 (0.06-0.2)	0.08	0.1	0.8
11	24	1.8	119	-17	0.7 (0.4-0.9)	0 (0-0.1)	0.04	0.3	0.9
10	12	4.0	77	-16	0	0.08 (0-0.9)	0.05	0.2	0.8
6	43	3.3	105	-15	0.1 (0-0.2)	0.09 (0.07-0.1)	0.11	0.1	0.7
20	29	4.2	99	-15	0.4 (0.4-0.7)	0.08 (0-0.1)	0.04	0.3	0.6
17	10	1.7	94	-11	0	0.03 (0.02-0.06)	0.04	0.3	0.2
9	24	4.0	73	-10	0.6 (0.4-0.8)	0	0	0.6	1.2
12	12	2.9	70	-7	0	0.1 (0.06-0.2)	0.04	0.001	0.5
15	15	4.9	82	-4	0	0	0	0.8	1.0
3	13	2.6	77	-3	0	0	0	0.4	1.0
14	35	1.9	106	-2	0	0	0	0.9	0.8
13	8	2.9	60	-1	0.4 (0.1-0.5)	0	0	0.1	0.3
16	6	2.7	74	-1	0.1 (0-0.6)	0.1 (0-0.1)	0.04	0.2	0.7
4	21	0.5 <sup>b</sup>	71	4	0.6 (0.4-0.7)	0.04 (0-0.09)	0.02	0.001	0.001
18	13	2.8	58	5	0.1 (0-0.1)	0	0	0.3	0.6

<sup>a</sup>Minimum concentration.<sup>b</sup>Admission blood slide unavailable.

Normal values: plasma Hb, unavailable; urine Hb, 0; plasma haptoglobin, 0.32-1.90 g/L; plasma haemopexin, 0.5-1.0 g/L.

blood Hb concentrations obtained simultaneously. Haemoglobinaemia was not associated with blood Hb loss or with haemoglobinuria.

The median haptoglobin concentration was 0.3 g/L (range, 0.001–1.8) (Table). Haptoglobin concentrations were near or below the lower reference value in most subjects on all days. Five children (ID #9, 11, 14, 15, 20) with slightly higher haptoglobin concentrations on admission had a pronounced fall in haptoglobin concentration over the 72-h observation period, but their haemopexin concentrations remained unchanged.

The median haemopexin concentration was 0.8 g/L (range, 0.1–1.2) (Table). Haemopexin concentrations were evenly distributed on admission, but there was an association between decline in plasma haemopexin over 72 h and drop in blood Hb concentration ( $P = 0.05$ ).

#### Haemoglobinuria

Haemoglobinuria was detected in 14 of the 20 children (Table). The median urine Hb concentration was 0.06 g/L (range, 0–0.97) and only 2 specimens showed macroscopic haematuria. Multiplying the urine Hb concentration with urine output, the median loss of Hb in urine over 72 h in children with haemoglobinuria was 0.05 g (range, 0.03–0.24). The occurrence of haemoglobinuria was associated with a greater fall in blood Hb concentration (Fig. 2). The median drop in blood Hb was 17 g/L compared to 2.5 g/L in those without haemoglobinuria ( $P = 0.009$ ). Individual differences in the relationship between haemoglobinuria and blood Hb loss were observed, e.g., in ID #2 and #5 with similar blood Hb losses, a 4-fold difference in haemoglobinuria was detected (Table). Haemoglobinuria was associated with indices of intravascular haemolysis, i.e., the individual minimum level of plasma haptoglobin ( $P = 0.004$ ) and the reduction in plasma haemopexin over 72 h ( $P = 0.04$ ). Haemoglobinuria was, however, not related to percentage parasitaemia ( $P = 0.34$ ). In children with haemoglobinuria, the median daily urine output decreased linearly with increasing Hb excretion and was lower than in subjects without Hb in urine ( $P = 0.03$ ). No child developed anuria. There was no association between haemoglobinuria and quinine treatment ( $P = 0.91$ ). As an example of a severe haemolytic episode, Figure 3 illustrates the individual concentrations of blood Hb, plasma Hb and urine Hb obtained in ID #5.

#### RBC-bound complement C3c and IgG

The median level of RBC-bound complement fragment C3c was 202 mOD/10  $\mu$ L membrane volume/min (range, 152–325), and the median level of autologous IgG was 40 mOD/10  $\mu$ L membrane volume/min (range, 31–56). Both levels were significantly higher

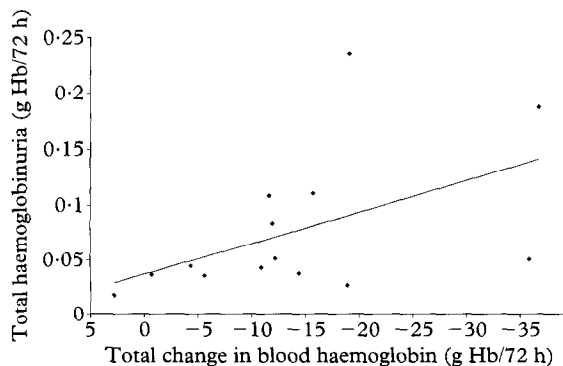


Fig. 2. The relationship between change in blood haemoglobin (Hb; g) and total haemoglobinuria (g) over 72 h in children with haemolysis due to acute *P. falciparum* malaria. Correlation coefficient,  $r_s = 0.50$ ;  $P = 0.07$ . For calculation of blood Hb (g) from blood Hb concentration (g/L), a blood volume of 0.075 L/kg bodyweight was assumed (KING, 1998).

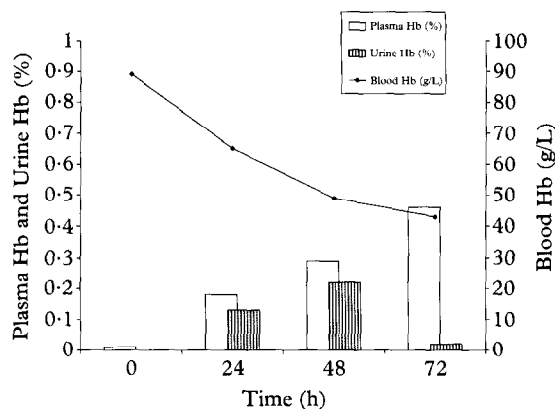


Fig. 3. Behaviour of total blood haemoglobin (Hb), plasma Hb and urine Hb over 72 h during a severe haemolytic episode due to acute *P. falciparum* malaria (ID #5). Total blood Hb concentrations (g/L) (line, right y-axis); concentrations (g/L) of plasma Hb (open columns, left y-axis) and urine Hb (stippled columns, left y-axis) are expressed as percent of blood Hb concentrations.

than those obtained from healthy controls ( $P < 0.05$ ) (Fig. 4). The change over time of both markers in 12 patients with major haemolysis is shown in Figure 4. On admission, the median C3c level was 210 mOD, i.e., 7-fold higher than controls. After 24 h, further

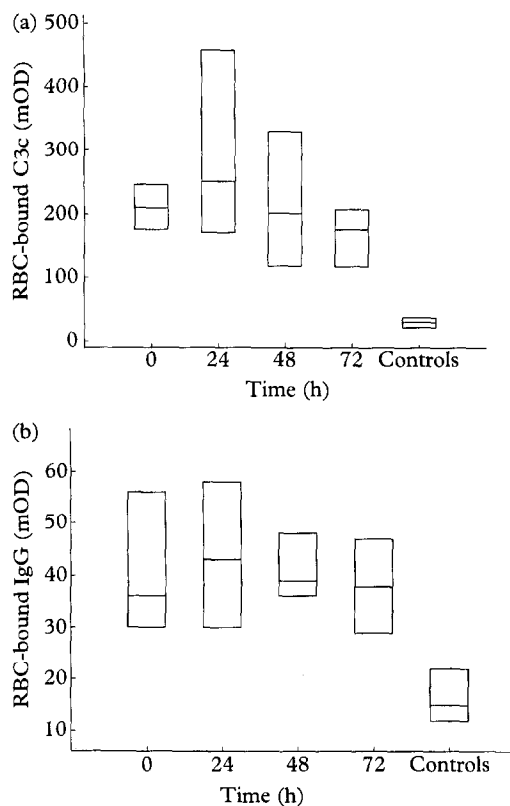


Fig. 4. Red blood cell (RBC)-bound complement fragment C3c (Fig. 4a) and IgG (Fig. 4b) in patients with major haemolysis due to acute *P. falciparum* malaria at 0, 24, 48 and 72 hours after the onset of treatment. Box plots with medians, lower and upper quartile values of 12 patients with severe haemolysis [haemoglobin (Hb) loss  $>10$  g/L] are shown. Average Hb loss was 8 g/L in the first 24-h period, 7 g/L in the second 24-h period and 6 g/L in the third 24-h period. The values of alkaline phosphatase activity of second antibodies to anti-human C3c and anti-human IgG are expressed as mOD<sub>405</sub>/10  $\mu$ L membrane volume/min. All differences to control values are statistically significant ( $P < 0.05$ ).

increases in bound C3c occurred with median levels 8.5-fold higher than controls. The increase in IgG was statistically significant over controls at all times but was less evident than the increase in C3c. Both levels declined over 72 h. The autologous IgG eluted from 4 malaria patients with major haemolysis was blotted on to membrane proteins containing aggregated band 3 isolated from zinc/BS3-treated RBCs. In 6 unfrozen samples [ID #1 (3 samples), 5, 9 and 19] the eluted antibodies reacted with the aggregated band 3 (not shown).

## Discussion

### *Parasitaemia and blood Hb loss*

Twenty episodes of high-level *P. falciparum* parasitaemia in children from an holoendemic area were prospectively investigated and quantitatively assessed for haemolysis. Only untransfused children with Hb > 50 g/L were studied. A wide range of blood Hb losses occurred during the observation period (Table). In all major haemolytic episodes the rapid blood Hb loss levelled out over 72 h as if self-limited, or, more likely, dependent on vanishing parasite load due to antimalarial therapy. The recorded Hb losses were not due to haemodilution by intravenous fluid replacement, and the indices of fluid balance were not significantly altered during the observation period. There was no relationship between the reduction in Hb and spleen size on admission. However, during antimalarial treatment the return to the circulation of RBCs pooled in the spleen could lead to an increase in Hb (WHITE, 1998), possibly explaining the increase in Hb observed in subjects with ongoing haemolysis (see for example ID #4 and #18; Table).

There was an overall linear relationship between maximum parasitaemia and the fall in blood Hb concentration (Fig. 1). However, the decrease in blood Hb was up to 9 times larger than the reduction in parasitaemia, possibly indicating the removal of variable numbers of uninfected cells (WHITE, 1998; JAKEMAN *et al.*, 1999). At parasite iso-density, some subjects (e.g., ID #8) experienced a pronounced drop in blood Hb whereas others (e.g., ID #15) were less affected (Table). The discrepancy between parasitaemia and drop in Hb may be due to selective removal of parasites from infected RBCs by macrophages ('pitting') (KUMARATILAKE *et al.*, 1994). Differences in immune status and phagocytic function, and variable sequestration in the deep circulation, may also have contributed (HO & WHITE, 1999).

Reticulocyte counts reflecting erythropoietic activity were not available. However, it was unlikely that a strong reticulocyte response would have modified the haematological status within the 72-h observation period, as reticulocytosis has been shown to develop only after parasite clearance (ABDALLA *et al.*, 1980; KURTZHALS *et al.*, 1997; NEWTON *et al.*, 1997), and only 2 children were cleared from parasites on the last blood collection.

### *Mode of haemolysis*

The relative shares of intravascular and extravascular haemolysis were semi-quantitatively determined by balancing the disappearance of Hb from blood with the increase of Hb in the plasma and the recovery of Hb in the urine. In intravascular haemolysis, Hb released into plasma binds to haptoglobin and haemopexin. When the plasma binding capacity is exceeded haemoglobinuria develops (PIMSTONE, 1972). In extravascular haemolysis, Hb is degraded to bilirubin within the reticuloendothelial system. Hence, in predominantly intravascular haemolysis, loss of blood Hb is expected to be recovered as Hb in the plasma and urine, whereas extravascular haemolysis may be indirectly determined as the difference between total blood Hb loss and the signs of intravascular haemolysis.

In the present study, the increases in plasma Hb were minor and <<1% of blood Hb concentrations, suggesting a low degree of intravascular haemolysis. Similar plasma Hb levels have been recorded in adults with *P. falciparum* malaria (DEVAKUL *et al.*, 1969). The total amount of Hb liberated into plasma over time is not easily determined, however, since the plasma Hb concentration is influenced by several factors, e.g., the rate of inflow of haemoglobin into plasma, organ uptake and metabolism of Hb and its carrier proteins, and the rate of renal reabsorption and excretion of Hb.

The plasma Hb did not show a linear relationship with haemoglobinuria or blood Hb loss. There may be several reasons for this. Plasma Hb analysis is very sensitive to in-vitro haemolysis by specimen collection and handling. Although measures were taken to prevent this, results of plasma Hb may have overestimated the degree of intravascular haemolysis. In addition, the spectrophotometric Hb reading may have been affected by individual differences in plasma turbidity caused by fibronectin precipitation in heparinized plasma. Furthermore, daily plasma Hb determinations were not able to capture brief episodes of intravascular haemolysis, and haemoglobinuria occurred despite the absence of recorded haemoglobinaemia (see for example ID #12; Table).

Haemoglobinuria was insignificant and only a minor portion of Hb lost from blood was recovered in the urine. Even in children with major haemolysis haemoglobinuria constituted <1% of blood Hb loss. Haemoglobinuria does not occur until the plasma binding capacity for Hb has been saturated and free Hb is filtered through the glomeruli (LOWENSTEIN *et al.*, 1961). The capacity to reabsorb Hb in the renal tubuli is minor (LATHEM, 1959), and the vast majority of Hb filtered through the kidneys is therefore recovered in the urine. It has been suggested that 0.3–0.6 g/L free Hb is the renal threshold for haemoglobinuria (LATHEM, 1959; WEATHERALL, 1996).

In our study, the geometric mean plasma Hb concentration in the children was 0.25 g/L, but this included both free and protein-bound Hb. It is thus plausible that plasma binding was a quantitatively important rescue mechanism for Hb in the children. The potential of haptoglobin and haemopexin to act as scavenger for Hb is, however, small, particularly in children. The release of 1 g Hb into plasma fully saturates the plasma binding proteins in adults (GIBLETT, 1968). In children, only a small fraction of this amount may be bound, since the plasma volumes are considerably smaller and the haptoglobin concentrations substantially lower.

The haptoglobin levels were commonly depressed already on admission. Similar levels have been reported in severe childhood anaemia (NEWTON *et al.*, 1997). Low levels may indicate ongoing intravascular haemolysis by asymptomatic malaria infection, but haptoglobin concentrations are age dependent and therefore low in young children (GIBLETT, 1968). In a few subjects with slightly higher admission haptoglobin, the levels decreased rapidly. Reduction in plasma haemopexin, a more specific but less sensitive marker for intravascular haemolysis, was more pronounced in episodes with severe haemolysis. This is consistent with haemopexin acting as second carrier protein for Hb in the plasma (MULLER-EBERHARD, 1970). Reductions in both haptoglobin and haemopexin were associated with haemoglobinuria and thus reflected intravascular haemolysis.

In conclusion, our data indicate that extravascular RBC removal, most likely by erythrophagocytosis, was the predominant mode of haemolysis, since disappearance of blood Hb corresponded to only small increases in plasma Hb, minor haemoglobinuria and limited haemoglobin binding capacity of the plasma in the children. The absence of multi-organ failure in the patients supported the predominance of extravascular

haemolysis, since such a complication would be likely if vast amounts of RBC lysed intravascularly. A linear association was found between blood Hb loss and haemoglobinuria (Fig. 2), indicating that intravascular haemolysis increased in parallel to blood Hb loss. No clinical threshold value for haemoglobinuria could, however, be identified from blood Hb loss. In addition, no relationship was found between parasitaemia and haemoglobinuria, despite the observed relationships between parasitaemia–blood Hb loss and blood Hb loss–haemoglobinuria.

Parasite densities were expressed as percentage infected RBCs, a more convenient unit in high parasitaemias than parasite counts per  $\mu\text{L}$  blood. The total parasite biomass will then depend on the total number of RBCs. Since practically all children were anaemic, we consider percent infected RBCs to be an acceptable relative estimate of biomass. To report absolute parasite counts involves counting parasites against white blood cells (WHO, 1991), but as white cell counts are age dependent (TRAPE *et al.*, 1994), a common standard count may not have been appropriate for all cohort children.

#### *Mechanism of extravascular RBC clearance*

Increased levels of the RBC-bound opsonins known to induce erythrophagocytosis, C3c complement fragment and IgG with specificity for aggregated band 3, were consistently observed in the patients. In children with major haemolysis, there was an initial increase in bound C3c and IgG that gradually declined with the elimination of parasites. Particularly the early increase in bound complement was prominent. Recognition of deposited complement by complement receptor type 1 (CR1) of the macrophage is a very efficient way to induce removal of effete or damaged RBCs by phagocytosis (see LUTZ, 1990 for review). Previous studies *in vitro* have shown complement activation by the surface of *P. falciparum*-infected RBCs (STANLEY *et al.*, 1984), and indicated that increased phagocytosis of ring-form infected human RBCs opsonized by non-immune serum was mostly dependent on complement fragment deposition and was mediated by CR1 on the macrophage (TURRINI *et al.*, 1992). *In vivo*, antibody deposition on infected and uninfected RBCs as well as antibody-mediated phagocytosis has been observed by numerous authors (FACER *et al.*, 1979; VERNES, 1980; FACER & BROWN, 1981; JEJE *et al.*, 1983; ABDALLA, 1988). Oxidative aggregation of band 3 and subsequent deposition of naturally occurring anti-band 3 IgG has been suggested to induce limited complement activation and C3b–C3c deposition on the RBC membrane (LUTZ, 1990). Recent data indicate that band 3 is aggregated in *P. falciparum*-infected RBCs (GIRIBALDI *et al.*, 2001). In the present study, eluted autologous IgG recognized aggregated band 3 present in RBC membranes prepared from zinc/BS3-treated RBCs (TURRINI *et al.*, 1994). This may suggest that the mechanism of haemolysis seen in the elimination of normal senescent or artificially modified RBCs (BEPPU *et al.*, 1990; LUTZ, 1990) may also operate in presumably non-immune children with malaria. The suggested mechanism is in agreement with previous data reported by FACER *et al.* (1979) showing that, in Gambian children with malaria, sensitization of RBCs with complement fragments was associated with anaemia.

#### *Clinical implications*

All major blood Hb drop occurred with limited signs of intravascular haemolysis, i.e., minor haemoglobinuria and increase in plasma Hb. Children without haemoglobinuria had a maximum blood Hb loss of 10 g/L (Table). A major Hb drop over a short time period could be deleterious for oxygen supply to vital organs, whereas a continuously low Hb concentration has less clinical implications owing to gradual physical adjust-

ment (CARSON *et al.*, 1988). Blood transfusions are life saving in anaemic children with clinical signs of respiratory distress, but should be used restrictively in areas without optimal blood-donor screening, given the risk for HIV transmission (LACKRITZ *et al.*, 1997). Under these circumstances, a urine dipstick test for Hb could help in the decision process to refrain from blood transfusion in a child admitted with clinical malaria and hyperparasitaemia. In the absence of haemoglobinuria, the risk for a significant drop in blood Hb concentration within 72 h was limited. Validation of this procedure, however, needs a larger sample size than that of the present study.

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## Announcement

### ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE Robert Cochrane Fund for Leprosy

The fund, in memory of the great leprologist Robert Cochrane, is administered by the Royal Society of Tropical Medicine and Hygiene. It is used to finance up to three travel fellowships each year to a maximum value of £1000 each.

The fund will support travel for

- Leprosy workers who need to obtain practical training in field work or in research
- Experienced leprologists to provide practical clinical training in a developing country

There is no restriction on the country of origin or destination providing the above requirements are met.

Applications must be made at least six months ahead of the proposed trip, sponsored by a suitable representative of the applicant's employer or study centre and agreed by the host organization. A short report on the travel/study should be submitted, within one month of the recipient's return. Application forms are available from the Administrator, Royal Society of Tropical Medicine and Hygiene, Manson House, 26 Portland Place, London, W1B 1EY, UK; fax +44 (0)20 7436 1389, e-mail mail@rstmh.org