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CARBOXYHEMOGLOBIN LEVELS IN KENYAN CHILDREN WITH *PLASMODIUM FALCIPARUM* MALARIA

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Abstract. Heme oxygenase (HO) is thought to be induced in severe malaria, but the pathophysiologic consequences have not been examined. It is induced by hemolysis, oxidative stress, and inflammation. It degrades heme, producing carbon monoxide (CO), which causes elevated levels of carboxyhemoglobin (COHb). In a prospective study of 1,520 children admitted to a Kenyan district hospital, COHb levels were no higher in children with malaria than with other infections. The COHb levels in children with severe malarial anemia were higher than in other children with malaria, but significantly lower than in children with other causes of severe anemia such as sickle cell disease. Levels of COHb were not significantly higher in children with cerebral malaria or in those dying of malaria. These results do not support a systemic increase in HO activity in malaria compared with other infectious diseases, but the roles of HO and CO in malaria require further study.

INTRODUCTION

It has recently been reported that expression of heme oxygenase-1 (HO-1) is increased in individuals dying of *Plasmodium falciparum* malaria.[1–3] Heme oxygenase degrades heme, producing biliverdin, iron, and carbon monoxide (CO), the latter binding to hemoglobin to form carboxyhemoglobin (COHb).[4–6] Levels of COHb are raised in hemolytic diseases such as sickle cell disease[7,8] and were high in a single case of cerebral malaria.[9] The roles of HO-1 and CO are postulated to be protective because they have potent anti-inflammatory effects in diverse settings. Increased HO-1 expression reduces heme-induced inflammation[10]; HO-1 activity and CO inhibit the expression of lipopolysaccharide-induced pro-inflammatory cytokines and promote the expression of anti-inflammatory cytokines[11,12]; CO rescues lung from damage by ischemia-reperfusion injury in HO-deficient mice;[13] and human HO deficiency is characterized by vulnerability to oxidative and inflammatory damage,[14] both of which occur in children with severe malarial anemia.[15,16] However, the formation of COHb reduces the oxygen delivering capacity of blood and may aggravate tissue hypoxia and acidosis seen in severe malaria in children. Furthermore hemozoin, a polymer of sequestered heme produced by *P. falciparum*, is resistant to degradation by HO and may interfere with breakdown of hemoglobin.[17]

To investigate the role of CO in *P. falciparum* malaria, we measured concentrations of COHb in children admitted to hospital with malaria, and compared them to children admitted with other diseases and those who had recovered from malaria.

MATERIALS AND METHODS

From June 2001 through November 2001, 1520 children more than six months old were admitted to Kilifi District Hospital. Kilifi is located on the coast of Kenya, where malaria is holoendemic and almost exclusively caused by *P. falciparum*. The predominantly rural population of the area has been described elsewhere.[18] Ethical approval was obtained from the Kenya Medical Research Institute ethics committee and children participated in the study after informed consent was obtained from a parent or guardian.

Demographic details, history, and examination findings by a clinician from the Kenya Medical Research Institute were recorded on a standardized proforma for all patients. Admission venous blood samples (prior to any blood transfusion) were examined for malaria parasites (thick and thin films stained with 10% Giemsa) and the number of pigment containing cells was counted in each of 200 monocytes, neutrophils, and erythrocytes. A full blood count using a Coulter Counter (MDII 18; Coulter Electronics, Ltd., Luton, United Kingdom) and venous blood gas analysis for COHb, oxyhemoglobin (OxyHb), reduced hemoglobin (RHb), pH, and base excess, using a co-oximeter (IL-682; Instrument Laboratory, Warrington, United Kingdom) were performed. Further investigations including radiology, cerebrospinal fluid analysis, bacterial cultures, repeat blood films, and hemoglobin electrophoresis were performed as necessary to reach a primary diagnosis for each patient.

A diagnosis of malaria was only made in children with a peripheral parasitemia and no other cause for the illness after review of all clinical and laboratory data. Severe malaria was defined as malaria with the presence of prostration, deep breathing, or severe malarial anemia (hemoglobin level $\leq$5 g/dL). Cerebral malaria was defined by a Blantyre coma score $\leq$2 or inability to localize a painful stimulus.[19] All children were treated according to local guidelines.[20] Additional diagnoses such as anemia or febrile convulsions contributing to the presentation, were recorded as secondary diagnoses.

An additional group of 50 children, who were well following outpatient treatment with lapudrine and dapsone for mild malaria, had hemoglobin and COHb concentrations measured at the same time that blood was taken on day 7 to confirm clearance of parasitemia. These children did not have quantitative assessment of malaria pigment on blood films.

Statistical analysis was performed using SPSS version 11.5 for the PC (SPSS, Inc., Chicago, IL). Central tendency for normally distributed variables was expressed as mean with standard deviation, and median with interquartile range for non-normal distributions. Student's *t*-test and one-way analysis of variance were used to compare means. Kendall's tau-b

test (non-parametric) was used for all correlations. A P value < 0.05 was considered significant. Further analysis to determine the effect of covariates on COHb was performed using stepwise linear regression. The effect of dichotomous variables, adjusted for the influence of other fixed factors and covariates, was assessed using general linear model regression analysis. Covariates of interest were included in the first fit of the model if significantly correlated with COHb ($P < 0.10$), then excluded stepwise if they failed to have a significant ($P < 0.05$) overall effect on the model.

RESULTS

Of 1,520 subjects, final diagnoses were available for 1,512, of whom 850 (56.2%) had a diagnosis of malaria, 277 (18.3%) had a diagnosis of anemia (Table 1), and 126 (8.3%) had malaria with severe anemia. Some patients had more than one diagnosis, giving a total of 2,124 diagnoses. In 1,477 children (97.2%), the level of COHb was >2%; the maximum reported for non-smokers in the United Kingdom.[21] More than 90% of the children were anemic (1,318 of 1,464 children in whom hemoglobin concentration was measured; hemoglobin level <11 g/dL in children <7 years old and <12 g/dL in children ≥7 years old).

There were significant positive correlations between COHb and age, spleen size, liver size, mean red blood cell volume, OxyHb, pH, and base excess (Table 2). There were significant inverse correlations between COHb and axillary temperature, white blood cell count, hemoglobin concentration, and RHb. Although these correlations were statistically significant, most of the correlation coefficients were very low, suggesting that these factors explained little of the overall variation in COHb. Carboxyhemoglobin was not significantly associated with conscious level, capillary refill time, or the proportion of malaria pigment–containing cells. Linear regression analysis showed that there were significant independent associations of COHb with age, hemoglobin concentration, mean red blood cell volume, pH, RHb, spleen size (all $P < 0.001$), and liver size ($P = 0.007$).

Mean COHb in children with malaria was not significantly different from children with other primary diagnoses except patients with sickle cell disease, in whom COHb was significantly higher (Table 1). There was no significant difference in mean levels of COHb in children with parasitemia on admission (n = 894, mean = 4.22%, SD = 1.49%) compared with those without parasitemia (n = 568, mean = 4.15%, SD = 1.57%). Malaria pigment was assessed in 1,481 subjects and the mean COHb was not significantly different between those with detectable pigment (n = 652, mean = 4.16%, SD = 1.29%) and those without (n = 829, mean =4.25%, SD = 1.66%). The mean COHb was significantly ($P < 0.001$) higher in subjects with severe anemia (n = 208, mean = 4.81%, SD = 1.76%) than in those without severe anemia (n = 1,256, mean = 4.10%, SD = 1.45%). Mean COHb in those with a primary diagnosis of severe malarial anemia (n = 109, mean = 4.43%, SD = 1.51%) was significantly ($P = 0.012$) lower than in those with severe anemia associated with other primary diagnoses (n = 67, mean = 5.13%, SD = 1.89%). Mean COHb in jaundiced subjects (n = 29, mean = 5.52%, SD =1.47%) was significantly ($P = 0.002$) higher than in those without jaundice (n = 1,488, mean =4.19%, SD = 1.50%).

TABLE 1
Carboxyhemoglobin (COHb) levels in the most common clinical diagnoses*

	n†	Mean COHb (95% CI)‡	SD	P§
Malaria	850 (689)	4.08 (4.00–4.18)	1.44	–
Anemia	277 (33)	5.09 (4.43—5.75)	1.85	0.20
Gastroenteritis	181 (89)	3.83 (3.53–4.14)	1.37	1.00
LRTI	178 (93)	4.12 (3.84–4.40)	1.37	1.00
Malnutrition	150 (81)	4.05 (3.77–4.32)	1.24	1.00
Febrile convulsion	142 (15)	4.98 (4.14–5.82)	1.51	0.88
Sickle cell disease	32 (22)	6.56 (5.71–7.41)	1.91	< 0.001
PUO	29 (27)	4.15 (3.58–4.74)	1.48	1.00
Soft tissue infection	27 (14)	3.69 (3.03–4.34)	1.15	1.00
Burns	27 (25)	3.46 (3.02–3.90)	1.08	0.40
Epilepsy	24 (16)	5.09 (4.32–5.86)	1.44	0.54

* CI = confidence interval; LRTI = lower respiratory tract infection; PUO = pyrexia of unknown origin.
† Total. Values in parentheses are the number with a primary diagnosis only.
‡ For primary diagnosis only.
§ Compared with malaria by one-way analysis of variance using the *post hoc* Tamhane T2 test.

Malaria without other coexisting illness. Six hundred eighty-nine patients had a primary diagnosis of malaria and no other coexisting illness. Median age was 26 months (interquartile range = 16–42 months) and 668 (97.0%) had COHb levels >2.0%. The mean hemoglobin concentration was 7.64 g/dL (SD = 2.37) and 109 (16.3%) of 669 children were severely anemic. In these children, COHb was significantly correlated with spleen size, oxyHb, mean cell volume, and pH (all $P < 0.001$). There were inverse correlations of COHb with temperature ($P = 0.044$), RHb, hemoglobin concentration (both $P < 0.001$), white blood cell count ($P = 0.008$), and log (parasite density) ($P = 0.033$). There was no significant correlation between COHb and age, liver size, capillary refill time, conscious level, base excess, or proportion of pigment containing neutrophils, monocytes, or erythrocytes. Stepwise linear regression analysis showed that only hemoglobin concentration, mean red blood cell volume, RHb, and pH were significantly (all $P < 0.001$) independently associated with COHb. Only six children with a primary diagnosis of malaria were jaundiced, with a mean COHb of 4.16% (SD = 1.30%), which was not significantly higher than in the other children with malaria (n = 681, mean = 4.08%, SD = 1.37%).

Severe malaria. The mean COHb of 4.31% (SD = 1.42%) in children with severe malaria was significantly higher ($P = 0.038$) than in other children with malaria (n = 566, mean = 4.01%, SD = 1.36). The independent associations of the different manifestations of severe malaria with COHb were compared using general linear model regression analysis (Table 3). Severe anemia was associated with a significantly higher mean COHb and deep breathing was associated with a significantly lower mean COHb. Mean levels of COHb in those with prostration, cerebral malaria, or dying of malaria were not significantly different from those in other children with malaria.

Adjusted analysis. To confirm that differences in COHb observed between groups were due to the diagnosis rather than other factors, further analysis was performed to adjust for the effect of significant laboratory and clinical variables. The covariates included in each initial model were age, RHb, mean cell volume, and pH. In models used to compare COHb between groups with different manifestations of malaria, log

TABLE 2
Association of carboxyhemoglobin (COHb) with clinical and laboratory characteristics in univariate analyses*

	n	Mean (95% CI)	SD	Median	Interquartile range (range)	Correlation coefficient†	*P*
Age (days)	1,520			739	440–1367	0.056	0.001
Temp (°C)	1,518	38.1 (38.0–38.1)	1.34		37.1–39.1	–0.043	0.014
Spleen (cm)	1,520			0	0–1 (0–20)	0.099	< 0.001
Liver (cm)	1,520			0	0–0 (0–10)	0.040	0.047
Capillary refill (sec)	1,520			1	0–2 (0–9)	0.005	0.79
BCS	1,520			5.00	5–5 (0–5)	–0.10	0.63
COHb (%)	1,520	4.21 (4.13–4.29)	1.52		3.10–5.00	–	–
RHb (%)	1,520			24.9	11.9–41.4	–0.316	< 0.001
OxyHb (%)	1,520			70.1	54.1–82.5	0.265	< 0.001
Hb (g/dL)	1,464	8.03 (7.90–8.15)	2.53		6.33–9.90	–0.172	< 0.001
MCV (fL)	1,463	70.4 (69.9–70.9)	10.1		63.1–76.2	0.096	< 0.001
WCC (10^9/L)	1,464			12.1	8.80–16.7	–0.065	< 0.001
Parasites (/μL)	1,462			1,870	0–98,400	–	–
Pigment-containing neutrophils (/200)	1,479			0	0–2 (0–64)	0.004	0.853
Pigment-containing monocytes (/200)	1,481			0	0–6 (0–79)	–0.020	0.314
Pigment-containing erythrocytes (/200)	1,474			0	0–0 (0–179)	0.016	0.446
BE (mEq/L)	1,467			–7.70	–10.9–5.50	0.045	0.012
pH	1,467	7.35 (7.34–7.35)	0.095		7.31–7.40	0.158	< 0.001

* n = total number (for some subjects admission data on temperature, full blood count, parasite count, pH and base excess were unavailable); Cl = confidence interval of mean; SD, standard deviation; Temp = axillary temperature on admission; Spleen/liver = distance from palpable limit of organ to costal margin; BCS = Blantyre Coma Score; RHb = reduced hemoglobin; OxyHb = oxyhemoglobin; MCV = mean cell volume; WCC = white cell count; BE = base excess.
† Using Kendall's tau-b correlation.

(parasite density) was also included as a covariate. No adjustment was made for hemoglobin concentration or spleen and liver size because our hypothesis was that hemolysis would be the main cause of any differences in COHb. Adjusted analysis did not substantially change the results of any comparisons made with univariate analysis. However, if adjustment was also made for hemoglobin concentration, the mean COHb in severe malaria was not significantly different from that in other children with malaria, indicating that the degree of anemia accounts for the difference between these groups.

Comparison with control children. In 50 well and aparasitemic children who had completed outpatient treatment of mild malaria, the mean COHb was 1.13% (SD = 0.73%), which was significantly lower than in subjects admitted to hospital with a primary diagnosis of malaria ($P < 0.001$). Only 4 (8%) of these children had COHb >2%. The mean hemoglobin concentration was 9.71 g/dL and no children had severe anemia. Carboxyhemoglobin was not significantly correlated with hemoglobin concentration (r = -0.18, $P = 0.072$).

TABLE 3
Carboxyhemoglobin (COHb) levels associated with clinical features in children with a primary diagnosis of malaria*

	n	Mean (95% CI) COHb	SD	*P*†	*P*‡
Primary diagnosis of malaria	689	4.08 (4.00–4.18)	1.44	–	–
Any features of severe malaria	123	4.31 (4.05–4.56)	1.42	–	0.012
Severe anemia	109	4.43 (4.15–4.72)	1.51	0.001	0.040
Deep breathing	50	3.82 (3.47–4.18)	1.26	0.002	0.021
Prostration	107	4.27 (3.98–4.56)	1.51	0.092	0.350
Cerebral malaria	58	4.17 (3.77–4.59)	1.57	0.792	0.503
Death	12	3.97 (3.00–4.93)	1.52	0.842	0.632

* CI = confidence interval.
† Using general linear model regression unadjusted for covariates.
‡ Using general linear model regression with initial covariates: age, temperature, reduced hemoglobin concentration, mean cell volume, white cell count, log (parasite density), and pH.

DISCUSSION

Levels of COHb in this study of children admitted to a rural Kenyan hospital were uniformly increased compared with levels reported in studies of children and adults in the United States and the United Kingdom, and levels in children who had recovered from mild malaria. The mean COHb was 4.2%, which was much higher than levels seen in American children (range = 0.6–3.9%, mean = 1.3%) and healthy, nonsmoking, adults in the United Kingdom (mean ~ 1%).[7,21] However, within our study of inpatients, increased levels of COHb were not associated with parasitemia or a primary diagnosis of *P. falciparum* malaria, and this does not support greater induction of systemic HO-1 activity in malaria compared with other conditions. We were able to assess and adjust for the effect on COHb of a number of potentially important physiologic parameters, but in fact these had a negligible influence on the mean levels of COHb associated with different illnesses.

There are a number of possible explanations for the high overall levels of COHb in our population. Levels of COHb are determined by both endogenous and exogenous sources of CO,[22] and environmental CO in smoke from traditional cooking methods can lead to high levels of COHb,[23] although the levels were lower in children who had recovered from mild malaria. In west African adults, COHb concentrations even higher than those seen in our populations have been reported.[24] In this study, 90% of the children were anemic and many children in this area of Kenya, even with iron deficiency anemia, have evidence of hemolysis and dyserythropoiesis,[25] both of which would be expected to increase endogenous CO production. The majority of our patients had in-

fectious illnesses requiring inpatient treatment and it is likely that some of the increased CO production was associated with sepsis.[26] Although levels of COHb were not significantly different in patients with primarily non-infectious illnesses such as epilepsy or burns, it is possible in some cases that infection triggered seizures and that there was secondary infection or a systemic inflammatory response associated with severe burns. This perhaps explains why levels of COHb in children at the end of outpatient treatment of mild malaria, without an active systemic inflammatory response, were dramatically lower than in any of the groups of inpatients.

In those with malaria, levels of COHb were not significantly different from those with other infections such as lower respiratory tract infection, gastroenteritis, and soft tissue infections; results were compatible with malaria generating a similar inflammatory response to other infections. Most of the manifestations of severe malaria were not associated with significantly different levels of COHb except for deep breathing, in which lower levels of COHb were most likely as a result of high ventilation rate,[22] and severe anemia, where higher levels were presumably due to hemolysis. However levels of COHb seen in subjects with malaria, and even severe malarial anemia, were considerably lower than in patients with sickle cell disease where the mean COHb of 6.56% is similar to values found in studies in the United States.[8,27] This difference may be explained by the differences in hemolytic rate in each condition. In adults with severe *P. falciparum* malaria, the mean red blood cell lifespan has been shown to be approximately 40 days,[28] which is substantially longer than in sickle cell anemia where it may be only 10–20 days.[29] The finding of increased expression of HO-1 in the brains, lungs, and livers of individuals dying of malaria[1–3] does not prove either systemic or local increases in HO-1 activity. It is possible for HO-1 to be induced but deprived of substrate, for example by sequestration of heme as hemozoin, or even for HO-1 activity to be inhibited. There is *in vitro* and *in vivo* evidence that hemozoin is resistant to degradation by HO-1 and its presence reduces CO production in mice with malaria.[17,30] In our study, parasitemia was inversely correlated with COHb in children with malaria, and we found no correlation between COHb and circulating levels of malaria pigment.

Any degree of reduction in HO-1 activity could be important in the pathophysiology of malaria through loss of protection against oxidative damage, and loss of the anti-inflammatory properties of CO. A relative deficiency of HO-1 activity may contribute to the oxygen radical production and oxidative stress which damages erythrocyte membranes in severe malarial anemia.[15,16] A reduction in CO production may promote the inflammatory process in severe malaria. It has recently been proposed that the combined action of CO and nitric oxide may be important in the development of tolerance to malaria by attenuating the systemic inflammatory response.[31] Insufficient CO production may shift the balance in favor of the inflammatory cascade.

In summary, this study suggests similar levels of HO activity in malaria to those seen in other infections, as measured by levels of COHb. Systemic levels of COHb in humans may not reflect CO production in individual tissues and further studies are warranted to determine whether there is a relative reduction in HO activity in malaria, which may be of importance in its pathogenesis.

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