

NITRIC OXIDE, MALARIA, AND ANEMIA: INVERSE RELATIONSHIP BETWEEN NITRIC OXIDE PRODUCTION AND HEMOGLOBIN CONCENTRATION IN ASYMPTOMATIC, MALARIA-EXPOSED CHILDREN

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Abstract: The cause of the anemia associated with chronic, intermittent, asymptomatic, low-level parasitemia in children in malaria-endemic endemic areas is not well understood. Nitric oxide (NO) decreases erythropoiesis, and it is likely an important mediator of anemia of chronic disease. Production of NO is decreased in acute uncomplicated and cerebral malaria, but it is increased in asymptomatic Tanzanian children (with or without parasitemia). We hypothesized that chronic overproduction of NO in these asymptomatic children contributes to the anemia associated with subclinical/subpatent malaria. In 44 fasting, asymptomatic, malaria-exposed, Tanzanian children, NO production (measured using fasting urine NOx excretion) was inversely associated with hemoglobin concentration (P = 0.03, controlling for age and gender). Using multiple linear regression, hemoglobin concentration was negatively associated with parasitemia (P = 0.005). After controlling for age and parasitemia, NO was no longer an independent predictor of anemia. One of the mechanisms of parasite-related anemia in such children may be through the adverse hematologic effects of parasite-induced NO production.

Anemia is extremely common in children in malaria-endemic areas of sub-Saharan Africa. Its etiology is complex with multiple overlapping causes such as malaria, iron deficiency, and hemoglobinopathies. Malaria is an important and often predominant cause. Two forms of malaria-associated anemia predominate: anemia associated with acute clinical episodes of malaria (or a history of such episodes), and anemia associated with the chronic, intermittent, asymptomatic, low-grade parasitemias found in up to 100% of children in endemic areas. In this latter group with asymptomatic parasitemia, the anemia is frequently out of proportion to the low level of parasitemia found, suggesting that it not mediated simply by direct destruction/hemolysis of parasitized red blood cells. The anemia of asymptomatic parasitemia is important since children may later develop severe anemia, both with and without subsequent episodes of acute clinical malaria.

Nitric oxide (NO) mediates a diverse array of physiologic and pathologic processes, and appears to be an important mediator of the protective immune response to all stages of Plasmodium infections. Cytokine-induced NO is known to decrease human erythropoiesis, and NO is likely an important mediator of the anemia of chronic disease in humans. We have recently shown that NO production and mononuclear cell expression of the inducible isoform of NO synthase (NOS2) are suppressed in Tanzanian children with uncomplicated clinical malaria and cerebral malaria, but are increased in healthy, asymptomatic, malaria-exposed children. Because this increased NO production/NOS2 expression in asymptomatic children is likely to be sustained, we hypothesized that overproduction of NO in these children (in part related to subclinical/subpatent infection with P. falciparum) contributes to the anemia associated with chronic subclinical/subpatent malaria. Therefore, we examined the relationship between production of NO and hemoglobin concentration in asymptomatic malaria-exposed Tanzanian children.

PATIENTS AND METHODS

Patients. Patients were those prospectively recruited as control children from the Paediatric Surgical ward at Muhimbili Medical Centre (MMC) in Dar es Salaam, Tanzania for a study examining the role of NO in severe and uncomplicated malaria. The protocol was approved by the College Research and Publications Committee at MMC and the Institutional Review Board of Duke University Medical Center. Informed consent was obtained in Kiswahili from all parents or guardians of study children. The children were asymptomatic, malaria-exposed children 6 months to 9 years of age, with no fever or history of fever within the last two weeks, a normal white blood cell count, and no acute illness (a fracture older than 1 week was permitted). All children were awaiting elective surgery for noninflammatory, nonmalignant disorders (mostly talipes and cleft lip repairs), resident healthy siblings, or children receiving bed rest for an uncomplicated long bone fracture of greater than 1-week duration. All children resided in malaria-endemic areas prior to admission, the majority being from Dar es Salaam, a city with low-moderate malaria transmission. Patients were subsequently categorized into 3 groups based on level of P. falciparum parasitemia: thick film positive (group 1); thick film negative, polymerase chain reaction (PCR) positive (group 2); and thick film negative, PCR negative (group 3).

Dietary control. Exogenous dietary nitrate ingestion can contribute significantly to urine and plasma nitrate levels, without indicating increased endogenous NO production. We therefore collected fasting samples using a previously described protocol validated in both children and adults at the MMC study site. Briefly, children were given a low nitrate dinner, and then they fasted overnight. The first morning void was discarded and following a distilled water challenge, the second fasting spot urine and venous blood were collected.

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249
Sample collection and parasitology. Urine was collected into isopropanol to prevent bacterial nitrate reduction. Venous blood was collected into tubes containing EDTA. Hemoglobin concentration and white blood cells counts were measured using a Coulter (Miami, FL) counter. Thick films were stained using Field’s stain A and B, and 50 oil-immersion fields were examined by an experienced microscopist. Thin films were stained with Giemsa stain. The numbers of parasites per 200 white blood cells were counted from thick films. Parasitemia (per microliter of whole blood) was calculated from the automated white blood cell count. Whole blood was preserved as 15-μl blood blots on 3M (St. Paul, MN) blotting paper for PCR analysis, which was performed as described previously. Briefly, fragments of two P. falciparum genes were amplified: the CD36-binding domain of sequesterin, and the 19-kD carboxy terminal fragment of merozoite surface protein-1 (MSP1). If a first amplification was negative, a second nested amplification was performed. Parasitemia was categorized into 3 groups: thick film positive (group 1); thick film negative, PCR positive (group 2); and thick film negative, PCR negative (group 3).

Nitrate and nitrite quantitation. Nitric oxide is rapidly oxidized to the stable metabolites nitrate and nitrite in vivo. Urine NOx (nitrate plus nitrite) was measured using bacterial nitrate reductase coupled with the Griess reaction as described. Because of variability in urine concentration, spot samples were normalized, expressing nitrate concentration as a function of creatinine (i.e., as NOx:creatinine ratios).

Statistical analysis. Multiple linear regression analysis was first used to model the relationship between hemoglobin concentration (dependent variable) and urine NOx excretion (using logarithm of urine NOx:creatinine ratio) controlling for age only (an important determinant of both hemoglobin concentration and NO production). To examine the confounding effect of parasitemia, we then incorporated parasitemia category (see Patients) into the regression model.

RESULTS

Of the 44 fasting, asymptomatic children recruited, 22 were thick film negative, PCR negative (group 1, mean age = 4.1 years); 13 were thick film negative, PCR positive (group 2, mean age = 4.9 years); and 9 were thick film positive, PCR positive (group 3, mean age = 5.1 years). The mean hemoglobin levels were 11.1 g/dl (range = 8.1–13.1) g/dl, 11.4 g/dl (range = 9.6–14.5), and 9.2 g/dl (range = 6.8–12.8) in each group, respectively. Sixty-one percent of all children were anemic by World Health Organization criteria (< 7 years of age: hemoglobin level < 11.0 g/dl; ≥ 7 years of age: hemoglobin level < 12.0 g/dl). Hemoglobin was inversely associated with NO production, as measured by urinary NOx excretion (Figure 1) (r = 0.093). However after controlling for age and parasitemia, there was no longer an independent association between hemoglobin and NO production (P = 0.37, regression coefficient = −0.40 (95% CI = −1.23 to 0.43)]. In the regression model including age, NO and parasitemia, there was a significant independent inverse effect of parasitemia on hemoglobin. Hemoglobin concentration was significantly lower in category 3 than in category 1 (P = 0.005, regression coefficient = −1.92, 95% CI = −3.22 to −0.6).

DISCUSSION

The basal mononuclear cell NOS2 expression and high constitutive NO production demonstrated in healthy malaria-exposed asymptomatic Tanzanian children is striking, since leukocyte NOS2 expression is rarely found in asymptomatic adult residents of non-malarious regions. Although age-related changes in leukocyte NOS2 expression may be important, it appears to be at least partly related to parasitemia in that NO production was higher in those children with patent parasitemia than in those without patent parasitemia on thick film examination. Parasitized red blood cells and parasite products are known to stimulate mononuclear phagocyte production of NO in vitro. While the background anemia seen with such high frequency in sub-Saharan African children is multifactorial, there are several lines of evidence that parasite-induced mononuclear cell NOS2 expression and overproduction of NO found in such children might contribute to the anemia of subclinical/subpatent malaria. In vitro studies show that tumor necrosis factor TNF- and interferon-γ-induced suppression of human hematopoiesis is in part mediated by NO. Furthermore, in studies using human CD34+ bone marrow cells, low to moderate concentrations of NO selectively inhibit erythroid colony growth and enhance myeloid colony growth. Higher concentrations of NO inhibit growth of both erythroid and myeloid colonies. In vivo studies in rodents also support a role for parasite-induced NO in the pathogenesis of anemia. The anemia found in rodent Trypanosoma brucei infection correlates directly with increased bone marrow NO production, and is largely reversed with NOS inhibitors. In a P. berghei rodent malaria model, NO production in response to P. berghei-specific T cell transfer was...
associated with the development of an anemia that was also reversible with NOS inhibitors (Good M, unpublished data).

Nitric oxide also alters cellular iron metabolism, and it likely contributes (through its effects on iron metabolism) to the anemia of chronic diseases. Iron deficiency itself is a major cause of anemia in malaria-endemic areas. However, interpretation of biochemical markers of iron status is very difficult in such areas, since these markers are altered in malaria and other clinical and subclinical infections. In coastal Kenya, no relationship was found between serum measurements of iron status and absence of stainable iron stores in the bone marrow, and there was no relationship between iron concentration and parasitemia. For these reasons, measurements of serum iron status were not performed in this study.

We have demonstrated that NO production is negatively associated with hemoglobin concentration in asymptomatic malaria-exposed Tanzanian children, independent of the known effect of age on hemoglobin concentration. After controlling for parasitemia, NO production alone was no longer an independent predictor of anemia in these children. As others have found in such children, we have shown that parasitemia was associated with the severity of anemia. Although it is not possible to establish a causal relationship, the findings are consistent with the hypothesis that one of the mechanisms of parasite-related anemia in asymptomatic malaria-exposed children is through the adverse hematologic effects of parasite-induced NO production.

Any adverse influence of NO on hematopoiesis is likely to result from the effects of sustained NO production over days-weeks in response to chronic parasitemia. Production of NO is likely to fluctuate longitudinally in response to the longitudinal fluctuations in parasite density known to occur in asymptomatic parasitemia. A limitation of a cross-sectional study such as ours is that parasitemia and NO production measured on any one day will not necessarily reflect mean parasitemia and mean levels of NO production to which the bone marrow has been exposed in preceding weeks. Although there was no history of fever in the two weeks prior to recruitment, it is possible that the hemoglobin levels we measured could also have been influenced by past intercurrent episodes of acute clinical malaria. Nevertheless, despite the potential for both of these considerations to dilute any association between hemoglobin concentration and spot measures of NO production/parasitemia, such an association was demonstrable.

Because systemic NO production in coastal Tanzanian children is decreased in both acute uncomplicated and cerebral malaria, we do not hypothesize that NO is involved in the anemia that develops acutely during clinical malaria in these two groups of children. Acute hemolysis and impaired bone marrow response are important in this setting. In our previously described study groups with acute clinical malaria with parasitemia > 10,000 trophozoites/μl and with cerebral malaria, NO production was significantly lower than in groups of healthy, asymptomatic malaria-exposed children with subclinical or subpatent parasitemia. Moreover, we were unable to detect a difference in NO production within the cerebral malaria group with or without severe anemia (mean urine NOx:creatinine ratios of 0.18 [n = 22] and 0.12 [n = 58], respectively; \( P = 0.19 \), or in acute clinical malaria between those severely anemic with a hemoglobin level < 5 g/dl and those with a hemoglobin level > 5 g/dl (mean urine NOx:creatinine ratios of 0.24 [n = 8] and 0.23 [n = 45], respectively. However, our numbers of conscious febrile children with severe malarial anaemia were small. Recent evidence suggests that severe malarial anemia in febrile conscious children may be a distinct immunologic entity characterized by low interleukin-10 (IL-10) production, in contrast to the much higher IL-10 levels found in both cerebral malaria and acute uncomplicated malaria. Because reduced IL-10 may lead to increased NO production, it will be important to examine NO production/NOS2 expression in larger numbers of febrile conscious children with severe malarial anemia. In children with all of these syndromes of acute malaria it is plausible that the ability of the bone marrow to adequately replenish erythrocytes after acute clinical malaria could be blunted by high levels of NO production and mononuclear cell NOS2 expression re-established during convalescence.

Longitudinal studies in larger numbers of patients with both asymptomatic and acute clinical malaria, including those assessing the hematologic response to antimalarials, will help us understand the role of NO in malarial anaemia further. However, longitudinal assessment of NO production will require careful adherence to protocols for dietary control to enable NOx levels to be interpretable.

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