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.\textsuperscript{1} We have recently shown in Tanzanian children that systemic NO production and mononuclear cell (MNC) expression of the inducible isoform of NO synthase (NOS2) are inversely related to malaria disease severity. Although suppressed in cerebral malaria, systemic NO production/MNC NOS2 expression was universally increased in healthy, asymptomatic malaria-exposed children with or without parasitemia.\textsuperscript{2} Nitric oxide production/MNC NOS2 expression was higher in those asymptomatic children with parasitemia found on thick film examination, suggesting that NO production in these children may in part be due to the known ability of parasites to induce macrophage NOS activity.\textsuperscript{3,4} However, the majority of the asymptomatic children with constitutive expression of NOS2 were thick film negative, raising the possibility that NOS2 expression was due to parasitemia below the detection limits of microscopy, subclinical infection with other pathogens, or age-related physiologic NOS2 expression in childhood.

Age appears to influence not only the acquisition of clinical immunity to malaria but also the susceptibility to and clinical manifestations of severe malaria.\textsuperscript{5-7} Because NO production has been linked with host protection in malaria,\textsuperscript{2,8} we sought to determine the effects of age and parasitemia on NO production. We measured NO metabolites and leukocyte NOS2 expression in 45 fasting, asymptomatic, malaria-exposed children of different ages, stratifying parasitemia by thick film and polymerase chain reaction (PCR) analysis. Although NO production was significantly higher in thick film–positive children than in thick film–negative children, after adjusting for age and gender, we were unable to detect a difference in NO production in thick film–negative children between those who were PCR positive and PCR negative. The relationship between age and NO production was determined using a generalized additive model adjusted for the effects of gender and parasitemia. Production of NO using all three measures was highest in infancy, decreasing after the first year of life, and then increasing again after 5 years of age. This pattern of age-related NO production is the reverse of the pattern of age-related morbidity from cerebral malaria in coastal Tanzanian children. Elevated production of NO in both infants and older children may be related to age per se and malaria infection respectively, and may be one of the mediators of the anti-disease immunity found most commonly in these two age groups.

**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, a city with low-moderate intensity of malaria transmission, for a study examining the role of NO in cerebral and uncomplicated malaria.\textsuperscript{2} 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 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. Patients were subsequently categorized into 3 groups based on level of *P. falciparum* parasitemia: thick film positive, PCR positive (group 1); thick film negative, 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\textsuperscript{9,10} 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.\textsuperscript{9,10} Briefly, children were given a low-nitrate dinner, and then fasted overnight. The first morning void was discarded and following a distilled water challenge, the second fasting spot urine and venous blood were collected.
Sample collection and parasitology. Urine was collected into isopropanol to prevent bacterial nitrate reduction. Venous blood was obtained into tubes containing EDTA. White blood cell 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. 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. Fragments of two *P. falciparum* genes were amplified: the CD36-binding domain of sequestrin, and the 19-kD carboxy terminal fragment of merozoite surface protein-1 (MSP-1).

If a first amplification was negative, a second nested amplification was performed. To extract DNA, dot blots were incubated in 1% saponin overnight, washed 3 times in phosphate-buffered saline, then heated in 200 μl of 5% (w/v) Chelex 100 (Sigma, St. Louis, MO) in distilled water for 15 min at 56°C and 10 min at 100°C. A 2.5-μl aliquot of the supernatant was used for a 20-μl primary PCR amplification; 1.5 μl of the primary amplification was used in the nested amplification. The PCR conditions were as follows: sequestrin primary amplification: primers RS (CTC ACA TAT TAG ATG AAC ATG) and seq nest A1 (TCT ACA TGA TCT TTA TGT ATA TCT); reaction denatured for 1 min at 95°C, then amplified at 95°C for 15 sec, 45°C for 15 sec, and 72°C for 1 min for 27 cycles. Sequestrin secondary amplification: primers RS and R3A3 (ACT ATA AAT TGC ATC TCG); reaction as for the primary amplification, with an annealing temperature of 44°C for 25 cycles; the MSP-1 primary amplification: primers MSP19 S1 (GCT TGG CAA ATT ACT CAC CAA); reaction as above with annealing temperature of 50°C and total 25 cycles; the MSP-1 secondary amplification: primers MSP19 S (AAC ATT TCA CAA CAC CAA TGC) and MSP19 A (ACT GCA GAA AAT ACC ATC GAA); reaction as above with an annealing temperature of 50°C for 25 cycles. Products were separated by electrophoresis on a 2% agarose gel, stained with ethidium and visualized by UV transillumination. Parasitemia (per microliter of whole blood) was determined. To extract DNA, dot blots were incubated in 1% saponin overnight, washed 3 times in phosphate-buffered saline, then heated in 200 μl of 5% (w/v) Chelex 100 (Sigma, St. Louis, MO) in distilled water for 15 min at 56°C and 10 min at 100°C. A 2.5-μl aliquot of the supernatant was used for a 20-μl primary PCR amplification; 1.5 μl of the primary amplification was used in the nested amplification. The PCR conditions were as follows: sequestrin primary amplification: primers RS (CTC ACA TAT TAG ATG AAC ATG) and seq nest A1 (TCT ACA TGA TCT TTA TGT ATA TCT); reaction denatured for 1 min at 95°C, then amplified at 95°C for 15 sec, 45°C for 15 sec, and 72°C for 1 min for 27 cycles. Sequestrin secondary amplification: primers RS and R3A3 (ACT ATA AAT TGC ATC TCG); reaction as for the primary amplification, with an annealing temperature of 44°C for 25 cycles; the MSP-1 primary amplification: primers MSP19 S1 (GCT TGG CAA ATT ACT CAC CAA); reaction as above with annealing temperature of 50°C and total 25 cycles; the MSP-1 secondary amplification: primers MSP19 S (AAC ATT TCA CAA CAC CAA TGC) and MSP19 A (ACT GCA GAA AAT ACC ATC GAA); reaction as above with an annealing temperature of 50°C for 25 cycles. Products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized by UV transillumination. 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 nitrite and nitrate 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. Plasma nitrate and nitrite were measured by capillary electrophoresis of plasma ultrafilterate. Nitrate and nitrite were added and expressed as total NOx. Plasma NOx levels were also corrected for differences in renal function and NOx handling among study children and expressed as plasma NOx:creatinine ratios.

Nitric oxide synthase type 2 immunoblot analysis of peripheral blood mononuclear cell extracts. Cellular extracts were prepared and analyzed for NOS2 antigen content by an immunoblot as previously described. To quantitate NOS2 antigen, immunoblot band density was measured using a densitometer (Molecular Dynamics, Inc., Sunnyvale, CA). Sufficient mononuclear cells were available from only 21 children in the study group.

Statistical analysis. Multiple linear regression analysis was used to model the relationship between parasitemia (categorized into 3 groups: thick film positive; thick film negative; PCR positive; and thick film negative, PCR negative) and log-transformations of each of the three measures of NO production (urine NOx:creatinine, plasma NOx, and plasma NOx:creatinine ratio) adjusting for the potential confounding effects of age and gender. The relationship between age and each of the three measures of NO production was determined using a generalized additive model adjusting for the effects of gender and parasitemia (categorized into the three groups). Cubic smoothing splines were used to present this relationship graphically as a line of best fit. Because the data were adjusted for the effects of gender and parasitemia, residual values were plotted rather than raw data points. Residual values indicate the difference between the observed values and the values predicted by a model that does not include the effect of age, but which does include the effects of gender and parasitemia. We then examined whether a nonlinear relationship was a better explanation of the data than a simple linear relationship. Nonlinearity was evaluated by testing the deviance increase that occurred if a linear term was used to model the predictor instead of the smoothing term. This deviance was assumed to have a chi-square distribution with degrees of freedom associated with the non-linear part of the relationship. Since there was no evidence for nonlinearity for the relationship between age and immunoblot band density (P = 0.56), this relationship was examined using multiple linear regression.

RESULTS

Of the 45 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); 10 were thick film positive, PCR positive (group 3, mean age = 5.1 years).

After adjusting for age and gender, we were unable to detect a difference in NO production between groups 1 and 2 (P = 0.57, 0.94, and 0.78 using urine NOx excretion, plasma NOx, and plasma NOx:creatinine, respectively). Production of NO was significantly higher in group 3 than in group 1 (P = 0.02, 0.02, and 0.005 for each measure of NO production, respectively) (Figure 1).

The relationship between age and NO production was not linear. Figure 2 shows this relationship using unadjusted data. Figure 3 shows the relationship between age and each measure of NO production using a generalized additive model adjusted for the effects of gender and parasitemia. Production of NO using all three measures was highest in infancy, decreasing after the first year of life, and then increasing again after 5 years of age. The model generated for each measure of NO production was significantly different from linearity (P = 0.02, 0.04, and 0.01 for urine NOx:creatinine (Figure 3A), plasma NOx (Figure 3B), and plasma NOx:creatinine (Figure 3C), respectively).

In contrast to the U-shaped age-related changes in NO
Figure 1. Production of nitric oxide (NO) stratified by parasitemia category. Production was measured using urine NOx excretion (urine NOx:creatinine µmol/µmol) (right axis) and plasma NOx (left axis); bars show the mean ± SEM. Group 1 are thick film-negative and Plasmodium falciparum polymerase chain reaction (PCR) negative (n = 22); group 2 are thick film negative, PCR positive (n = 13); and group 3 are thick film positive (n = 10).

Figure 2. Relationship between nitric oxide (NO) production and age. Production measured by urine NOx excretion (urine NOx:creatinine µmol/µmol). Data are stratified by parasitemia category. Diamonds = thick film negative (neg), polymerase chain reaction (PCR) negative; circles = thick film negative, PCR positive (pos); squares = thick film positive.

The changes in NO production with age in our malaria-exposed study group were striking. In two studies of non-fasting Japanese children in non-malaria endemic areas,\textsuperscript{15,16} urine NOx excretion was highest in infancy, decreasing progressively with age. A similar age-related decrease has been found with cerebrospinal fluid NOx concentrations in European children.\textsuperscript{17} Plasma NOx levels were not measured in any of these studies. In our study population from a malaria-endemic area, each of the three measures of NO production were also highest in infancy and decreased with age. However, in contrast to data from the non-malarious areas, NO production increased again after 5 years of age. Because the same U-shaped pattern was observed for plasma NOx as for the urine NOx:creatinine and plasma NOx:creatinine ratios, and because changes in urine creatinine concentration with age were minimal, our results are unlikely to be related to an age-related effect on the denominator (creatinine) used to standardize NOx concentrations, or to changes in renal NOx handling with age.

The elevated NO production in infancy may not be due to increased NO production by circulating MNC. Although numbers were small, MNC NOS2 expression was lowest in infancy and increased with age. It is possible that elevated NO production in infancy may be derived from hepatocytes, tissue macrophages, or other cell types. However, the increase in NO production after 5 years of age may be derived from the increased MNC NOx expression we found with age, which in turn may be related to the effects of ongoing malaria exposure in childhood. Larger numbers of subjects are needed for a more definitive analysis.

Leukocyte NOS2 expression is rarely found in asymptomatic adult residents of non-malarious regions.\textsuperscript{12,18} Because NO production is higher in those children with patent parasitemia than in those without parasitemia on thick film examination, the universal mononuclear cell NOS2 expression and high constitutive NO production found in malaria-exposed asymptomatic Tanzanian children\textsuperscript{2} is likely to be at least partly related to subclinical parasitemia.

We were unable to detect a difference in NO production between those thick film–negative children who had parasitemia demonstrable by PCR and those who were PCR negative, a finding that needs confirmation in larger numbers of children. A possible explanation for this is the presence of a threshold for parasite induction of macrophage NO in vivo. A threshold for induction of macrophage NOS activity by schizont extracts has been noted in in vitro studies.\textsuperscript{4} Similar findings have been found in studies of T cell activation. Low concentrations of Plasmodium antigen result in interleukin-2 (IL-2) production, T helper cell type 1 proliferation but no NO production, whereas higher concentrations of antigen result in high level NO production and decreased cell IL-2 secretion and cell proliferation.\textsuperscript{19} A further possibility is that any difference in NO production between children who are PCR positive and those who are PCR negative could have been masked by background NO production induced as a result of subclinical infection with other endemic pathogens. Furthermore, changes in parasite-induced NOS expression and excretion of NO metabolites may lag behind longitudinal fluctuations in parasitemia,\textsuperscript{20} making correlation of NO
production and parasitemia at any one time problematic, particularly at low parasitemias.

In most rodent models of malaria, NO has more of an anti-disease, host-protective effect than a direct anti-parasitic effect.1 In humans, systemic NO production is also associated with protective rather than disease-producing responses.2 Because NO down-regulates tumor necrosis factor, the major cytokine responsible for malarial fever,3 and also down-regulates cytokine-induced expression of the endothelial adhesion molecules involved in parasite cytoadherence,4 we and others5 have speculated that increased NO production in malaria-exposed children may protect parasitemic children from clinical disease. Although the age distribution varies with intensity of malaria transmission, morbidity and mortality attributable to malaria in coastal sub-Saharan Africa is low in early infancy, peaks in early childhood, and then decreases in older children.6-9 In coastal Tanzania, we also found this age-related pattern of morbidity/mortality following further analysis of our previously described group of children with cerebral malaria (mean age

Figure 3. Relationship between nitric oxide (NO) production and age generated using a generalized additive model and cubic spline smoothing operation. Measures of NO production used to model this relationship were urine NOx:creatinine (A), plasma NOx (B), and plasma NOx: creatinine (C). For each measure of NO production, the vertical axis indicates residual values, these being the difference between the observed values and the values predicted by a model that does not include the effect of age, but which does include the effects of gender and parasitemia. The bold line is the fitted model (with dotted lines indicating the 95% confidence interval). For each measure of NO production, the model differs significantly from linearity ($P = 0.02, 0.04$, and $0.01$, respectively).

Figure 4. Relationship between age and mononuclear cell expression of nitric oxide synthase type 2 (NOS2). The NOS2 antigen quantitated by immunoblot band density and expressed as a percentage of the human colon cancer cell line DLD1 (positive control) ($r^2 = 0.18, P = 0.055$).
tibodies to toxin in older children have been invoked as community/malaria tolerance noted in infancy and older children mechanisms of asymptomatic parasitemia/anti-disease im-

Tanzania (mean age in both infancy and older children may be related to age production, may also be important. Elevated production of NO in both infancy and older children may be related to age per se and malaria infection respectively, and may be an additional mediator of the anti-disease immunity found most commonly in these two age groups.

In contrast to cerebral malaria, high rates of asymptomatic parasitemia are found in infancy and older children. The mechanisms of asymptomatic parasitemia/anti-disease immunity/malaria tolerance noted in infancy and older children are not clear. Maternally derived IgG in infancy antibody to toxin in older children have been invoked as potential mediators. Age-dependent changes in innate immunity, including those we have shown with NO production, may also be important. Elevated production of NO in both infancy and older children may be related to age per se and malaria infection respectively, and may be an additional mediator of the anti-disease immunity found most commonly in these two age groups.

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References


Figure 5. Age-related frequency of cerebral malaria in children recruited at the same time and same study site in Dar es Salaam, Tanzania (mean age = 3.9 years). See Anstey and others for details of subject recruitment.


