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Nitric Oxide in Tanzanian Children with Malaria: Inverse Relationship between Malaria Severity and Nitric Oxide Production/Nitric Oxide Synthase Type 2 Expression

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Summary

Nitric oxide (NO)-related activity has been shown to be protective against *Plasmodium falciparum* in vitro. It has been hypothesized, however, that excess NO production contributes to the pathogenesis of cerebral malaria. The purpose of this study was to compare markers of NO production [urinary and plasma nitrate + nitrite (NO_x)], leukocyte-inducible nitric oxide synthase type 2 (NOS2), and plasma TNF-α and IL-10 levels with disease severity in 191 Tanzanian children with and without malaria. Urine NO_x excretion and plasma NO_x levels (corrected for renal impairment) were inversely related to disease severity, with levels highest in subclinical infection and lowest in fatal cerebral malaria. Results could not be explained by differences in dietary nitrate ingestion among the groups. Plasma levels of IL-10, a cytokine known to suppress NO synthesis, increased with disease severity. Leukocyte NOS2 antigen was detectable in all control children tested and in all those with subclinical infection, but was undetectable in all but one subject with cerebral malaria. This suppression of NO synthesis in cerebral malaria may contribute to pathogenesis. In contrast, high fasting NO_x levels and leukocyte NOS2 in healthy controls and asymptomatic infection suggest that increased NO synthesis might protect against clinical disease. NO appears to have a protective rather than pathological role in African children with malaria.

Malaria continues to be a major cause of morbidity and mortality in African children, being responsible for approximately 1–2 million deaths each year (1, 2). The nature of the protective immune response to *Plasmodium falciparum* is not fully understood. Similarly unclear is the pathogenesis of cerebral malaria, a major cause of malaria mortality in Africa. Both parasite sequestration within cerebral blood vessels and cytokine-mediated immunopathology are thought to be important (3).

Proinflammatory host cytokines such as TNF have protective antiparasitic effects in vitro (4). There is evidence, however, that excessive TNF production contributes to the pathogenesis of cerebral malaria (CM[1]; 5–7). Because TNF is a potent inducer of inducible nitric oxide synthase type 2 (NOS2) (8–10), more recent interest has focused on a possible similar dual role for nitric oxide (NO) in malaria. NO-related species mediate inhibition of malaria parasites by hepatocytes and monocytes in vitro (11–14). The role of NO in the human protective immune response to *P. falciparum* in vivo, however, has not been determined. Investigators have hypothesized that in CM, high local concentrations of TNF induce excessive synthesis of NO in

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[1] *Abbreviations used in this paper:* AP, asymptomatic parasitemia; CM, cerebral malaria; CMCR, CM with complete recovery; CMDS, CM complicated by death or neurological sequelae, cr, creatinine; FE_{NOx}, fractional excretion of nitrate and nitrite; HC, healthy controls; NO, nitric oxide; NOS2, nitric oxide synthase type 2; NO_x, sum of nitrate and nitrite concentrations; UM, uncomplicated clinical malaria; uNO_x, urinary NO_x.

Published August 1, 1996

cerebrovascular endothelial cells, and that this NO alters neurotransmission, causing profound but reversible coma (15).

NO is rapidly oxidized to the stable inorganic nitrogen oxides nitrite and nitrate in vivo (16, 17). Measurement of these metabolites has been used to demonstrate cytokine-inducible NO synthesis in humans (18). To assess the relationship between NO production and malaria disease severity, we prospectively measured NO metabolites and leukocyte NOS2 in five groups of Tanzanian children with and without cerebral malaria. Our results indicate that rather than being increased in cerebral malaria, NO production is inversely proportional to disease severity. We demonstrate in humans an in vivo correlation between increased NO production and protection from a human pathogen, as well as decreased NO production and severity of clinical infection.

Materials and Methods

Patients. This study was performed at Muhimbili Medical Centre (MMC), Dar es Salaam, Tanzania, from May 1994 to January 1995. The protocol was approved by the College Research and Publications Committee at MMC and the Institutional Review Board at Duke University Medical Center. Informed consent was obtained in Kiswahili from all parents or guardians of study children. Five groups of children 6 mo to 9 yr old were prospectively recruited from the pediatric and surgical wards: (*a*) healthy controls (HC), had no fever within the last 2 wk, no parasites on thick blood film, normal white cell count, and no acute illness (fracture older than 1 wk permitted); (*b*) asymptomatic parasitemia (AP) was defined as per healthy control, except for the presence of *P. falciparum* parasitemia on thick blood film; (*c*) uncomplicated clinical malaria (UM) was defined as a febrile illness with *P. falciparum* parasitemia of $\geq$10,000 trophozoites/μl, no history of convulsions, no other evident cause of fever, fully alert, normoglycemic, and without severe respiratory distress; (*d*) CM with complete recovery (CMCR) was defined as unarousable coma (2) with a Blantyre coma score (19) of $\leq$2 persisting for $\geq$30 min after the last convulsion, no other cause of coma evident from clinical or CSF analysis, any level of *P. falciparum* parasitemia on thick film examination, and recovery without neurological sequelae; and (5) CM complicated by death or neurological sequelae (CMDS) was defined as for CMCR, but with coma complicated by death or neurological sequelae on discharge. Patients with malaria were managed according to standard MMC guidelines.

Dietary Control. Exogenous dietary nitrate ingestion can contribute significantly to urine and plasma nitrate levels (20, 21). A protocol using a low nitrate dinner plus an overnight fast, followed by fasting morning spot urine collections for nitrate/creatinine measurement, gives results comparable to 24-h urine samples collected after 1–2 d of a low nitrate diet (21). We therefore used this method of dietary control in children in the HC and AP groups. Few of the commonly eaten local foods in Tanzania are high in nitrate (22). For their evening meal, subjects consumed their usual hospital diet and were then asked to fast overnight. Nitrate-free distilled water was provided with the evening meal and for overnight use. The morning after an overnight fast, the first urine specimen was discarded. After a distilled water challenge, the second fasting morning urine and venous blood were obtained. A 24-h dietary history of the previous day's food and fluid intake was also documented.

To confirm the adequacy of the control subject's dietary nitrate restriction, we placed a subset of 18 HC and AP children on a supervised standardized low nitrate diet (distilled water, white bread, baked chicken, and white rice cooked in distilled water) for 24 h. Urine samples were collected after a ward evening meal and overnight fast as described above, as well as after a 24-h low nitrate diet and a second overnight fast. The time since ingestion of last food and drink was documented in the UM and CM groups at the time of recruitment.

Sample Collection. Venous blood was obtained in EDTA and lithium heparin anticoagulant. Hemoglobin and white cell counts were measured using a Coulter counter. Thick blood films were stained using Field's stains A and B. Thin blood films were stained using Giemsa stain. Numbers of parasites per 200 white cells were counted from thick films. Parasitemia (per milliliter of whole blood) was then calculated from the automated white cell count. 50 oil immersion fields were examined by an experienced microscopist before a film was classified as negative. Urine was collected into isopropanol to prevent bacterial nitrate reduction. Plasma was aspirated from 3–4 ml of whole blood in lithium heparin anticoagulant within 30 min of collection and frozen at -70°C. Mononuclear cells were then separated by centrifugation over Ficoll/Hypaque (Lymphocyte Separation medium; Organon Technica, Durham, NC) and stored in liquid nitrogen until use. For logistical reasons, blood mononuclear cells could only be collected from those subjects recruited in the latter part of the study.

Nitrate and Nitrite Quantitation. Concentrations of nitrate and nitrite were measured by capillary electrophoresis of plasma ultrafiltrate, using a modification of the method of Leone and Kelm (23). Nitrate and nitrite concentrations were added and expressed as total NO_x. The final concentration of NO_x was corrected for trace amounts of nitrate and nitrite in the filter units and blood collection tubes, determined by capillary electrophoresis. Plasma creatinine was measured using an Ektachem autoanalyzer (Eastman Kodak Co., Rochester, NY). Because 60–73% of plasma nitrate is excreted renally (17, 24), nitrate is retained and plasma NO_x elevated in otherwise healthy humans with renal impairment (25). Plasma NO_x was therefore corrected for renal disfunction and expressed as plasma NO_x/creatinine (Cr) ratio.

Urine NO_x (uNO_x) was measured at a 1:19.3 dilution using *Pseudomonas oleovorans* nitrate reductase coupled with the Griess reaction, as described by Granger et al. (26). The possibility that urine at a 1:19.3 dilution inhibits bacterial nitrate reductase was excluded by running a standard curve in the 20 CM and UM urine samples that had the lowest NO_x concentrations. There was complete detection of added nitrate to 100 μM in 19 of 20 urines. Because of some variability of urine concentration, spot samples were normalized, expressing nitrate concentration as a function of creatinine concentration (Sigma Diagnostics, St. Louis, MO). Fractional excretion of NO_x (FE_{NOx}), the percentage of filtered NO_x that is excreted, was calculated by the formula: $FENOx = 100 \times (U/P\ NO_x)/(U/P\ creatinine)$.

Cytokine Measurement. Human IL-10, TNF-α, and IL-4 were measured with commercial reagents using standard sandwich ELISAs, as described previously (27). The capture/detecting biotinylated antibody pairs used were mAb-5817508/mAb-5817503 (Medgenix, Stillwater, MN) for TNF, mAb-M010/mAb-M-011 (Endogen, Inc., Boston, MA) for IL-10 and polyclonal Ab-P-451/ mAb-M450-B (Endogen) for IL-4. Assay sensitivities were 4.7 pg/ml for IL-10, 15.6 pg/ml for TNF, and 3.1 pg/ml for IL-4, respectively. Recombinant standards used for TNF, IL-10, and IL-4

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were rhIL-10 (*Escherichia coli*)-RIL-10-5 (Endogen), rhTNF (*E. coli*)-5817510 (Medgenix), and rhIL-4 (*E. coli*)-R-IL4-5 (Endogen).

NOS2 Immunoblot Analysis of PBMC Extracts. Cellular extracts were prepared and analyzed for NOS2 antigen content by immunoblot, as previously described (28). Immunoblots were done using a monoclonal anti-NOS2 antibody (Transduction Laboratories, Lexington, KY; 28) and ECL reagents from Amersham (Arlington Heights, IL). For known negative and positive extracts, we used untreated cells from the murine macrophage cell line J774, and J774 cells and cells from the human colon cell line DLD1 treated with rIFN-γ (200 U/ml; murine for J774 cells and human for DLD1 cells) and LPS (200 ng/ml) for 3 d. 50 μg protein from the human and murine cells was used in the individual lanes. An immunoblot for NOS2 was considered positive if a clear band was visible at 130–131 kD. To quantitate NOS2 antigen, immunoblot band density was measured using a densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Statistical Analyses. A general linear models approach (29) was used to model the relationship between markers of NO production and disease severity controlling for suspected covariates. Log transformations of the dependent variables were used to obtain normally distributed model residuals. Since there was little difference between the CMCR and CMDS groups, these groups were combined as a single CM group. Age, sex, h since last fed, chloroquine intake, parasite count, hemoglobin, and (in the case of plasma NO_x levels and urinary NO_x/creatinine ratio) the plasma creatinine level, were examined for covariance. The model that included all significant parameters was considered the "best" fit. Post-hoc disease category pairwise comparisons were done using a least squares means (LSM) approach (29). For each *t* test, significance was evaluated using a Bonferroni adjustment for multiple comparisons (i.e., $a_{ij} = 0.05/6 = 0.0083$ for each H_0: LSM [*i*] = LSM[*j*]). Pairwise comparisons of the differences between NO_x/creatinine ratios performed on consecutive days were performed using the Wilcoxon Rank Sum test. Differences among groups in the proportion with detectable PBMC NOS2 were assessed by chi-square analysis followed by pairwise comparisons using Fisher's exact test (significance set at $P < 0.0083$). The relationship between cytokine levels and disease severity was measured using the Kruskal-Wallis test followed by pairwise Mann-Whitney *U* tests. For this analysis, the HC and AP groups were combined. For statistical purposes, cytokine levels below the sensitivity of the assay were assigned a value of half the lower limit of detection.

Results

Subjects. There were 191 children in the study population. Mean age and weight, and sex distribution were similar for each study group (Table 1). Of the 50 children in the diet-controlled groups (HC and AP), 29 (22 HC, 7 AP) were healthy children awaiting elective surgery for noninflammatory and nonmalignant disorders (mostly talipes and cleft lip repairs) or their resident siblings, and 21 (18 HC, 3 AP) were receiving bed rest for a fractured long bone of greater than 1-wk duration. As expected, mean hemoglobin was inversely related to disease severity. Eight children with severe anemia (hemoglobin range = 3.1–5 g/dl) were included in the UM group. None of these eight had severe respiratory distress, as manifest by intercostal indrawing or deep breathing. Otherwise, all 55 children in the UM group had uncomplicated malaria as defined by The World Health Organization (WHO; 2). No children with UM died. The CM groups included >90% of children with WHO-defined CM admitted to MMC during the study period. Of the 36 children in the CMDS group, 26 died,

Table 1. *Baseline Characteristics of Study Groups*

Characteristic	HC	AP	UM	CMCR	CMDS
Number*	40	10	55	50	36
Mean age (years)	4.1	5.1	3.1	3.6	3.4
(range)	(0.5–9.6)	(0.7–8.4)	(0.5–7.8)	(0.6–7.2)	(0.7–7.2)
Mean weight (kg)	13.8	14.4	12.0	12.4	12.5
Median time since admission (h)	n.a.	n.a.	7.0	5.0	3.5
Mean time since last food (h)	13.0	13.0	5.6	16.2	17.7
Mean time since last fluid (h)‡	12.4	11.3	2.6	12.4	15.8
Chloroquine use in preceding week (%)	5	0	53	92	86
Sex (% male)	65%	60%	51%	44%	61%
Geometric mean parasitemia (trophs/μl)	0	595	45,624	23,963	44,691
Mean Hb (g/dL)§	11.2	9.2	7.3	6.4	6.3
Mean white cell count ($\times 10^9$/liter)¶	8.5	9.6	11.2	12.7	19.7

*Urine obtained in all but 2 of the UM group; number with plasma obtained: HC, 34; AP, 9; UM, 53; CMCR, 43; CMDS, 28.
‡Does not include distilled water ingested by HC and AP groups.
§Number for each group: HC, 35; AP, 9; UM, 55; CMCR, 48; CMDS, 31; normal range for hemoglobin (Hb) 6–23 mo of age, 10.5–13.5g/dL; 2–6 yr, 11.5–13.5 g/dL; 6–10 yr, 11.5–15.5 g/dL.
¶Number for each group: HC, 35; AP, 9; UM, 52; CMCR, 48; CMDS, 31.
Normal range for white blood cells 6 mo–2 yr of age: 6.0–17.5 × 10^9/liter; 3–6 yr: 5–15.5 × 10^9/liters; 7–10 yr: 4.5–13.5 × 10^9/liter.

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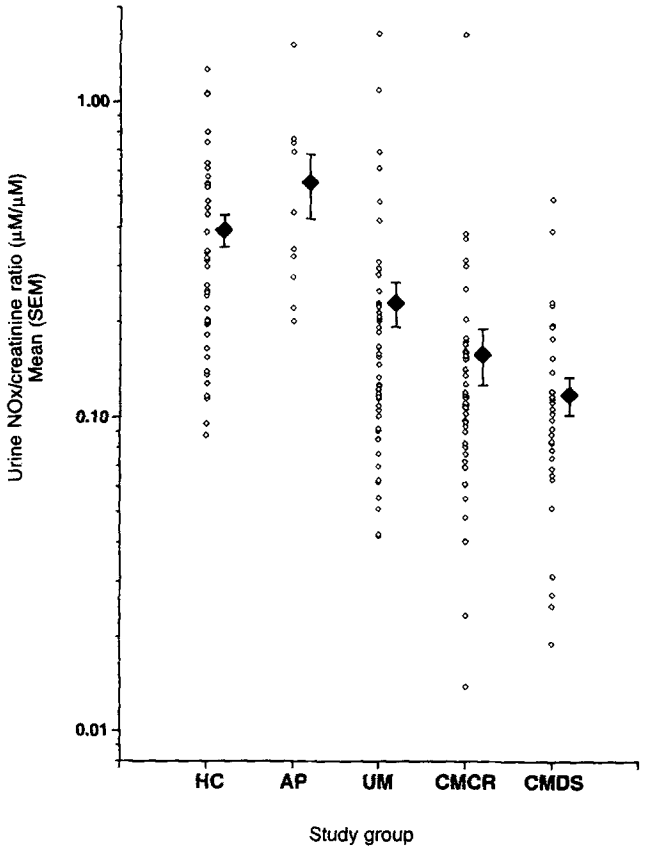


Figure 1. Urinary NO_x excretion in each study group. Data are expressed as urine NO_x/cr ratios (μM/μM, means ± SEM) on a logarithmic scale. Study groups are fasting HC (n = 40), fasting AP (n = 10), UM (n = 53), CMCR (n = 50), and CMDS (n = 36). The adjusted means for both the HC and AP groups differed significantly from each of the UM and CM groups (P = 0.0001). After Bonferroni adjustment for multiple comparisons, there was no significant difference in the adjusted means between the UM and CM groups (P = 0.216), or between the HC and AP groups (P = 0.0375).

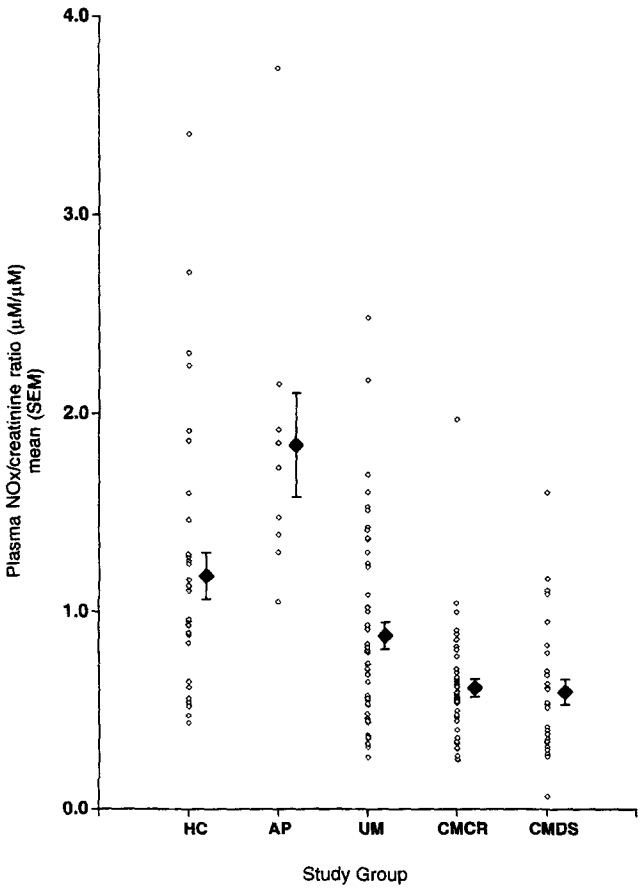


Figure 2. Plasma NO_x corrected for renal impairment. Data are expressed as plasma NO_x/cr ratios (μM/μM, means ± SEM). Study groups are fasting HC (n = 34), fasting AP (n = 9), UM (n = 53), CMCR (n = 43), and CMDS (n = 28). All disease category–adjusted means were significantly different from all others: HC vs. AP (P = 0.0008), HC and AP vs. UM and CM (P = 0.0001), and UM vs. CM (P = 0.0003).

giving an overall mortality rate for CM in this series of 30% (26 of 86). Neurological sequelae occurred in 10 of the 86 children with CM (12%), including 9 with cortical blindness and 5 with spastic quadriparesis. Convulsions were witnessed after admission in 77 and 72% of children in the CMCR and CMDS groups, respectively.

Urine and Plasma Nitrite and Nitrate Levels. Urinary nitrate excretion decreased in proportion to disease severity, being highest in those children with asymptomatic parasitemia and lowest in the CMDS group (Fig. 1). The best model fitting the relationship between the uNO_x/cr ratio and disease severity was the one that controlled for the covariates age, parasite count, and plasma creatinine (P = 0.0001). This model explained 50.3% of the overall variation in the uNO_x/cr. Nitrate excretion in both the UM and CM groups was significantly lower than in each of the HC and AP groups (P = 0.0001 for each comparison; Fig. 1).

Differences in mean plasma NO_x with (Fig. 2) and without (Table 2) correction for renal impairment paralleled the urine NO_x excretion results. Corrected and uncorrected plasma NO_x were highest in the AP group and lowest in the CM groups. The best model fitting the relationship between plasma NO_x and disease severity was the one that controlled for the covariates age, hours since fed, and the logarithm of creatinine (P = 0.0001). Relative to healthy controls, the least squares mean plasma NO_x was significantly lower in those with UM (P = 0.0007) and CM (P = 0.0001). The AP least squares mean plasma NO_x was significantly higher than in each of the HC (P = 0.0018), UM (P = 0.0001), and CM (P = 0.0001) groups (Table 2). Plasma levels of nitrite, the NO metabolite least influenced by diet (22), paralleled those of total NO_x (Table 2).

The best model fitting the relationship between plasma NO_x/cr ratio and disease severity was the one that controlled for age as the only significant covariate (P = 0.0001). This model explained 43.7% of the total variation in the plasma NO_x/cr ratio. Relative to HC, mean plasma NO_x/cr was higher in those with AP and lower in those with UM and CM (Fig. 2). All disease category–adjusted means were significantly different from all others (HC vs.

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Table 2. *Plasma NO Metabolite Concentrations and Renal Function in Each Study Group*

Characteristic	HC	AP	UM	CMCR	CMDS
Plasma (mean ± SEM*):					
Nitrate (μM)	30.8 ± 3.7	48.4 ± 7.4	25.5 ± 2.4	21.9 ± 1.6	29.0 ± 3.9
Nitrite (μM)	8.8 ± 0.6	9.4 ± 1.1	5.8 ± 0.3	6.6 ± 0.4	7.5 ± 0.7
NOx (μM)§	39.6 ± 3.9	57.8 ± 7.3	31.3 ± 2.4	28.5 ± 1.6	36.5 ± 4.1
Plasma creatinine*	34.8	32.0	36.9	51.8	69.1
mean (μmol/liter) (range)	(26–53)	(26–44)	(26–44)	(26–159)	(35–159)
Proportion having elevated plasma					
creatinine for age‡ (%)	0	0	0	21%	39%
Plasma NO_x corrected for renal impairment					
(NO_x/cr ratio) mean (μM/μM) ± SEM*¶	1.18 ± 0.12	1.84 ± 0.26	0.88 ± 0.07	0.62 ± 0.04	0.60 ± 0.06
FE_{NOx}	31%	31%	25%	24%	21%

*Number with plasma obtained: HC, 34; AP, 9; UM, 53; CMCR, 43; CMDS 28.
‡Normal range for plasma creatinine increases with age (30).
§The least squares mean plasma NO_x for the HC group differed significantly from those of the AP ($P = 0.0018$), UM, ($P = 0.0007$) and CM groups ($P = 0.001$). The AP least squares mean differed from that of both the UM and CM groups ($P = 0.0001$). After Bonferroni adjustment for multiple comparisons, the UM least squares mean was not significantly different from that of the CM group ($P = 0.0482$).
¶Least squares mean plasma NOx/cr ratios in all study groups were significantly different from all others: HC vs. AP ($P = 0.0008$), HC and AP vs. UM and CM ($P = 0.0001$), and UM vs. CM ($P = 0.0003$).

AP [$P = 0.0008$], HC and AP vs. UM and CM [$P = 0.0001$], and UM vs. CM [$P = 0.0003$]).

Uncorrected plasma NO_x levels were higher in the CMDS group relative to those in the CMCR and UM groups. However, even without correcting for renal impairment, plasma NO_x levels in CMDS were not elevated above the basal levels found in fasting healthy controls. Renal impairment, as evidenced by elevated plasma creatinine for age (30), was found only in children with CM (Table 2). After correction for renal impairment, mean plasma NO_x/cr in the CMDS group was the lowest of all groups. This suggests that impaired nitrate excretion rather than increased NO synthesis was the reason for the relative elevation of uncorrected plasma NO_x in CMDS.

The fractional excretion of NO_x (the proportion of filtered NO_x that is excreted) was decreased in those with CM, being lowest in the CMDS group (Table 2). Therefore, in CM, not only was glomerular filtration of nitrate reduced (as measured by increased plasma creatinine), but the filtered nitrate was more avidly reabsorbed. Both of these factors acted to elevate plasma NO_x levels in the CM group. Despite this, mean plasma levels of NO_x in CM were still lower than in HC.

Dietary histories from children in the HC and AP groups confirmed the intake of low nitrate foods (22, 31) for the evening meal before the overnight fast: 74% consumed brown beans, rice, and/or maize porridge, 16% fresh meat and rice, 8% chicken and rice, and 2% breast milk alone. Local foods potentially high in nitrate (spinach and cabbage) (22, 31) were eaten by only 3 of 50 children (all in the HC group). The mean fasting NO_x/cr ratio in urine samples obtained the next morning in these three children was 0.28, no higher than the group mean of 0.39. The only other vegetables consumed were the low nitrate vegetables potato ($n = 2$) and cassava ($n = 2$). Noncured fish was eaten by four children (three HC, one AP) with a mean fasting NO_x/cr ratio the next morning of 0.27, again less than the HC group mean. We confirmed that the ward evening meal was low in NO_x. In 18 children from the HC and AP groups, the mean fasting urine NO_x/cr ratio the morning after a normal ward evening meal (0.368) did not fall further after a standardized 24-h low nitrate diet (0.347; $P = 0.97$). Likewise, groundwater ingestion was not a confounding factor. NO_x concentrations in all samples taken from bedside water containers of 15 HC children were low (mean = 1.4 μM). Water samples drawn from five widely separated locations in Dar es Salaam was also relatively low in NO_x (mean = 10.9 μM [range = 8.1–12.9 μM]).

Chloroquine (to 5 μg/ml) has no effect on NOS activity in vitro (Hibbs, J.B., Jr., personal communication). Differences in previous chloroquine ingestion among the study groups (Table 1) did not influence urine or plasma NO_x when analyzed using linear modeling. There was no effect of a treatment course of chloroquine (10 mg/kg then 5 mg/kg, 44 and 52 h after commencing a low nitrate diet) on basal urine nitrate excretion in a group of five healthy adult volunteers. Mean fasting NO_x/creatinine ratios before and after chloroquine ingestion were 0.039 and 0.041, respectively ($P = 0.51$). There was no difference in NO_x levels between those children in the HC group with and without a long bone fracture, nor in the UM group with or without severe anemia.

Leukocyte NOS2 Antigen Content. To determine if leukocyte NOS2 was also modified in malaria, we measured

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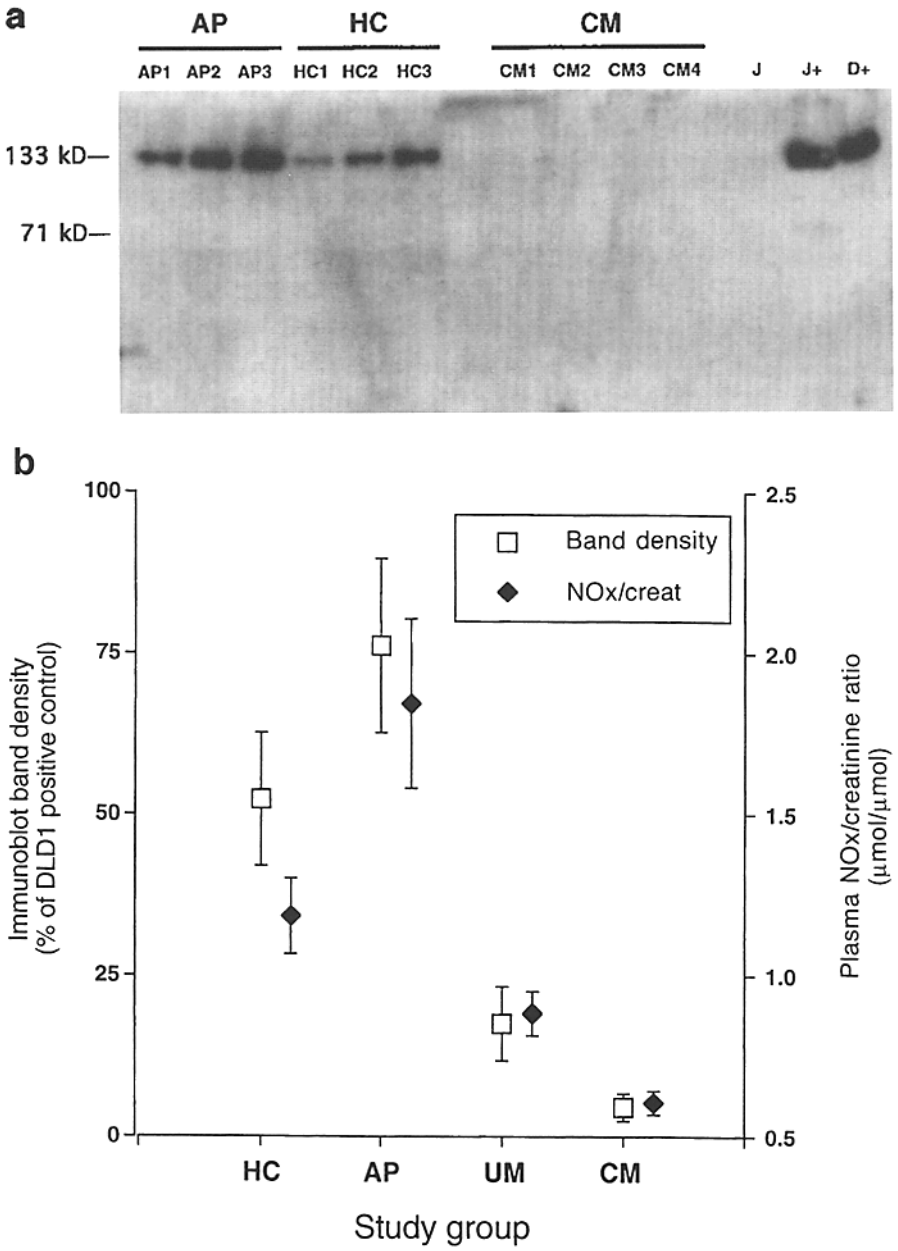


Figure 3. (*A*) Immunoblot analysis of mononuclear cells from study subjects for NOS2 expression. Blood mononuclear cells were isolated, and extracts were analyzed for NOS2 antigen content using an NOS2 mouse monoclonal anti-NOS2 antibody. The NOS2 antigen has a molecular mass of ~130 kD. Extracts from the murine macrophage cell line J774 and human colon cell line DLD1 were used as negative and positive controls. 50 μg of protein from the extracts were used in each lane. Immunoblots using isotype-matched (IgG2a) control Ig showed no reactivity. Only 1 of 18 CM patients was positive. In contrast, 15 of 15 healthy controls and 7 of 7 children with asymptomatic parasitemia showed a clear band at 130–131 kD. 5 of 14 patients with uncomplicated malaria were positive (four-group chi square: $P < 0.0001$). The HC and AP groups each differed significantly from the CM group ($P < 0.0001$ for each comparison) and UM group ($P = 0.0002$ and $P = 0.0071$, respectively). The difference between the CM and UM groups did not reach statistical significance ($P = 0.064$). *AP1-3*, samples from three children with asymptomatic parasitemia; *HC1-3*, samples from three healthy control children; *CM 1–4*, samples from four patients with cerebral malaria; *J*, J774 control; J^+, J774 cells cultured with LPS and IFN-γ; D^+, DLD1 cells cultured with LPS and human IFN-γ. (*B*) PBMC NOS2 antigen relationship to plasma NOx/cr ratios. NOS2 antigen was quantitated by immunoblot band densitometry. J774-negative control background was subtracted, and the results were expressed as a percentage of the DLD1-positive control density. Mean immunoblot densities in healthy controls ($n = 15$) and in asymptomatic parasitemia ($n = 7$) were significantly higher than in clinical malaria (UM; $n = 14$ and CM; $n = 18$). By four-group Kruskal-Wallis testing, $P < 0.0001$; HC vs. UM: $P = 0.0058$, AP vs. UM: $P = 0.0012$; HC vs. CM: $P < 0.0001$; AP vs. CM: $P = 0.0002$. The differences between HC and AP ($P = 0.098$) and between UM and CM ($P = 0.17$) were not statistically significant. PBMC NOS2 expression was closely associated with NO production in each study group as measured by the plasma NOx/creatinine ratio. *Squares*, immunoblot density (mean ± SEM); *diamonds*, plasma NOx/cr ratio (μM/μM) (mean ± SEM).

NOS2 antigen content by immunoblot analysis. PBMC lysates from only 1 of 18 CM patients (11 CMCR and 7 CMDS) contained a protein (130–131 kD) that reacted with the anti-NOS2 antibody. In contrast, NOS2 antigen was detected in all 15 HC and all 7 AP subjects in which cell lysates were tested. 5 of 14 cell lysates from UM children displayed the antigen on blot ($P < 0.0001$ by four-group chi square analysis). Fig. 3 *A* shows a representative immunoblot. For immunoblots using isotype-matched (IgG2a) control Ig, there was no positivity (data not shown). The HC and AP groups each differed significantly from the CM group ($P < 0.0001$ for each comparison) and UM group ($P = 0.0002$ and 0.0071, respectively). The difference between the CM and UM groups did not reach statistical significance ($P = 0.064$). Mean immunoblot band density in each group closely paralleled plasma NO_x/cr ratios (Fig. 3 *b*). NOS2 band density correlated significantly with plasma NO_x/cr ratios ($R = 0.319$; $P = 0.022$).

Plasma Cytokine Measurements. Plasma levels of both TNF and IL-10 correlated with disease severity, being highest in

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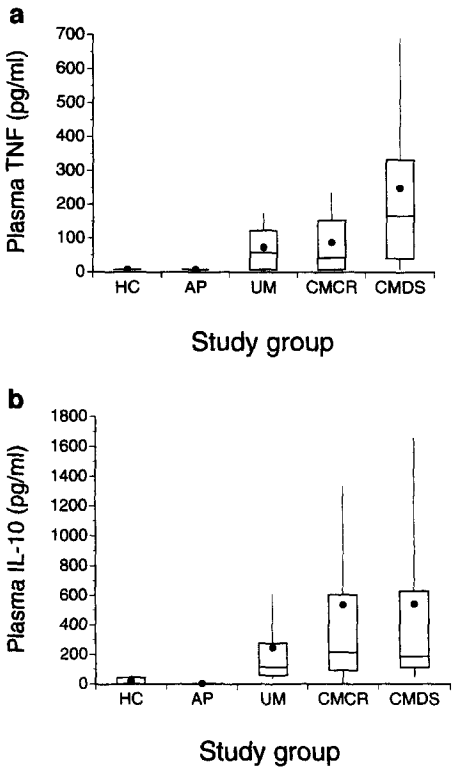


Figure 4. (*A*) Plasma TNF-α levels related to disease severity. Plasma levels of TNF correlated with disease severity. Number of samples assayed in each group were as follows: HC/AP, 27; UM, 39; CMCR, 42; and CMDS, 30. Box plots show the interquartile range. Medians and means are indicated by horizontal lines and circles, respectively. Vertical lines indicate the 10th–90th percentile ranges. TNF was significantly higher in the CMDS group than in the CMCR ($P = 0.0037$), UM ($P = 0.0009$), and HC/AP ($P < 0.0001$) groups. TNF was undetectable in all HC and AP subjects. Levels in HC and AP were significantly lower than in the UM, CMCR, and CMDS groups ($P < 0.0001$ for each comparison). There was no significant difference between the UM and CMCR groups ($P = 0.71$). (*B*) Plasma IL-10 levels related to disease severity. Plasma levels of IL-10 (pg/ml) correlated with disease severity. Number of samples assayed in each group were as follows: HC/AP, 27; UM, 39; CMCR, 42; and CMDS, 30. Box plots show the interquartile range. Medians and means are indicated by horizontal lines and circles, respectively. Vertical lines indicate the 10th–90th percentile ranges. Plasma IL-10 levels were higher in the combined CM group than in the HC/AP ($P < 0.0001$) and UM ($P = 0.032$) groups, and higher in the UM vs. HC/AP group ($P < 0.0001$).

those with fatal malaria (Fig. 4, *A* and *B*). TNF was significantly higher in the CMDS group than the CMCR ($P = 0.0037$), UM ($P = 0.0009$), and HC/AP ($P < 0.0001$) groups. TNF was undetectable in all HC and AP subjects, with levels significantly lower than in all the UM, CMCR, and CMDS groups ($P < 0.0001$ for each comparison). Plasma IL-10 levels were higher in the combined CM group than in the HC/AP ($P < 0.0001$) and UM ($P = 0.032$) groups, and higher in the UM vs. HC/AP group ($P < 0.0001$). Plasma IL-4 was undetectable in all CM samples tested ($n = 14$) and in all but one of the HC samples tested ($n = 12$).

Discussion

Levels of NO metabolites and leukocyte NOS2 expression were decreased in both UM and CM, indicating suppression of NO synthesis rather than excessive production in clinical malaria in children. The suppression of NO synthesis increased with disease severity. The known in vitro antiplasmodial effects of endogenous NO–related compounds suggest an in vivo protective role for NOS in malaria. IFN-γ and the parasite itself inhibit the development of the exoerythrocytic stage of the plasmodium species via the induction of NO within both murine and human hepatocytes (12, 13, 32, 33). Human monocytes have also been shown to inhibit erythrocytic stages of *P. falciparum* in vitro via secretion of nitrogen oxides (14). Suppression of NO-mediated protective immune responses after infection with *P. falciparum* may result in inadequate control of parasite replication, and may have contributed to the development of clinical malaria and CM in these children.

A protective role for NO-related activity in malaria is also supported by our finding that leukocyte NOS2 antigen and NO metabolite levels were highest in those with infection but no disease. Enhanced NO synthesis may have protected these children with low grade AP from clinically overt disease. However, longitudinal studies of host NO production will be required for final analysis. It was striking to observe the universal expression of NOS2 antigen in healthy control subjects. NOS2 expression in freshly isolated blood leukocytes of normal adults in the United States is rare (28, 28a). Possible explanations for our observations in cells from HC Tanzanian children include physiological high expression in childhood, genetic/racial differences, and subclinical or subpatent infection with malaria or other pathogens.

Several reports have described plasma NO_x levels in human malaria (34–38). It is difficult to extrapolate NO production from the NO_x levels measured in these studies because of absent or insufficient numbers of control subjects, inadequate control for the potential confounding effects of dietary nitrate ingestion, and lack of correction for renal impairment, altered FE_{NOx}, and altered volume of distribution of NO_x in severe malaria.

Despite the close correlation between leukocyte NOS2 antigen levels and plasma NO_x/cr ratios in the different groups, we considered potential artifacts that would have influenced the NO_x results. Our NO_x results cannot be explained by differences in nitrate/nitrite (NO_x) ingestion among the study groups. The Dar es Salaam water supply, particularly at the study site, was low in NO_x. The staple Tanzanian foods of rice, beans, and maize are all low in NO_x (22). The approach to dietary control in the HC and AP groups used in this study adequately excludes a confounding effect of dietary NO_x ingestion (21). Since the half life of nitrate after an oral nitrate load is 6–7 h (39), dietary nitrate would have been largely cleared in subjects in the HC, AP, and CM groups whose mean durations of fasting were two nitrate half lives or longer. NO_x excretion and plasma NO_x were less in the UM group, despite more

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Published August 1, 1996

recent potential NO_x ingestion than the fasting control group, and lower still in the CM groups despite comparable duration of fasting. It is theoretically possible that in severe malaria, compared with controls, a greater proportion of NO is metabolized via an as-yet unknown pathway to non–nitrate/nitrite end products. If true, measurement of NO_x would underestimate NO production. There is no evidence to date to support this notion, and this too would not account for the difference between the HC and AP groups.

Differences in renal nitrate excretion did not explain the differences in plasma or urinary NO_x levels among those in the HC, AP, and UM groups. Renal insufficiency caused higher plasma NO_x levels in the CMDS group than in the CMCR group (although not statistically significantly so). This difference disappeared once levels were corrected for renal impairment. If we had not analyzed a control group and corrected for renal impairment (25, 40, 41), this difference might have been incorrectly interpreted as increased NO synthesis in complicated vs. uncomplicated CM. Because the volume of distribution of NO_x (V_dNO_x) approximates extracellular fluid volume (41), plasma NO_x levels will be increased and NO formation will be overestimated in disease states characterized by extracellular fluid volume contraction. As others (42), we commonly observed dehydration in children with CM in this study. It is therefore likely that the V_dNO_x was reduced in the CMDS and CMCR groups, and that NO formation was overestimated in these groups. NO synthesis in CM may thus be even lower than we have calculated.

As others (5, 6, 43, 44) we have shown increased production in malaria of both the proinflammatory Th1 cytokine TNF and the antiinflammatory Th2 cytokine, IL-10. Levels of both cytokines correlated with disease severity. Decreased NOS2 expression and NO production in malaria could be caused by IL-10 (45–47) and other host Th2 cytokine responses. TGF-β also suppresses NO synthesis (48). TGF-β production in semiimmune African children with severe malaria is not yet known. We did not measure TGF-β in our plasma samples because of possible platelet contamination.

In addition to host factors, parasite factors may also be involved in modulating NO production after infection. *P. falciparum*–infected erythrocytes exhibit NO synthase activity (49). Parasite-derived products have been shown to both increase (49, 50) and decrease (51) NO production in vitro. Other intracellular protozoa such as *Leishmania major* are able to directly increase host TGF-β production, resulting in suppression of NOS2 expression and parasite survival (32). Strains of *P. falciparum* are known to vary in their ability to induce TNF production by human mononuclear cells, with some relationship to disease phenotype in the host (52). It is possible there is a similar strain heterogeneity in the ability to produce parasite NOS, or to induce or suppress human NOS2.

Suppression of NO production in clinical malaria and CM may have deleterious effects other than a possible impaired ability to suppress parasite development. TNFα, IL-1, and other proinflammatory cytokines are known to increase a number of human endothelial molecules involved in parasite cytoadherence and sequestration (e.g., ICAM-1, VCAM-1, and E-selectin [53–56]). NO decreases this endothelial activation. In human vascular endothelial cells, cytokine-induced expression of adhesion molecules such as VCAM-1, E-selectin, and to a lesser extent, ICAM-1 are all decreased by exogenous and endogenous NO (56) via inhibition of NF-κB (57). We speculate that decreased NO synthesis in malaria could thus cause loss of endothelial NF-κB inhibition, resulting in increased expression of endothelial adhesion molecules, increased parasite sequestration, and a greater likelihood of developing CM. Moreover, decreased NO production may also result in increased production of TNF. In rats, inhibition of NOS before endotoxemia results in a significant increase in circulating TNF and IL-6, and increased mortality (58). Inhibition of NO synthesis in malaria may thus cause a greater increase in circulating TNF and further upregulation of endothelial receptors involved in parasite sequestration.

Some have hypothesized that there is local upregulation of NOS2 activity in cerebrovascular endothelium, or that excessive local NO synthesis contributes to the coma of CM (15). However, based on our evidence for systemic suppression of NO synthesis in pediatric CM, we think that this is unlikely. Nevertheless, this cannot be excluded without doing detailed studies of brain specimens for NOS content and activity. Postmortem examination of brain tissue was not culturally or logistically possible in our study.

The mortality rate from CM remains 10–30%, despite prompt administration of the best available chemotherapy (42), and better therapies for treating CM are needed. NO synthesis appears to be suppressed in CM in African children, but the therapeutic implications of this require further study. Potential benefits of NO donors include the inhibition of parasite development (59) and a decrease in parasite cytoadherence (56), the process central to the pathogenesis of CM (60). Increased cerebral blood volume has been implicated in the pathogenesis of raised intracranial pressure in African children with CM (61, 62). It is not certain what effect NO donors may have on this process.

Because the pattern of disease phenotype and immune response varies with age and intensity of malaria transmission (63, 64), the role of NO in malaria needs to be determined in adult and pediatric populations from regions with different malarial epidemiology. Results from our study of African children in this region of moderately intense, stable malaria transmission demonstrate that NO production and NOS2 expression correlate with protective rather than disease-producing responses in malaria infection. We document an in vivo correlation between increased NO production/NOS2 expression and suppression of a human pathogen, as well as a correlation between decreased NO production/NOS2 expression and disease severity.

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