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INDUCIBLE NITRIC OXIDE SYNTHASE (NOS2) PROMOTER CCTTT REPEAT POLYMORPHISM: RELATIONSHIP TO *IN VIVO* NITRIC OXIDE PRODUCTION/NOS ACTIVITY IN AN ASYMPTOMATIC MALARIA-ENDEMIC POPULATION

CRAIG S. BOUTLIS, MAURINE R. HOBBS, ROBYN L. MARSH, MARY A. MISUKONIS, ARIANA N. TKACHUK, MOSES LAGOG, JENNIFER BOOTH, DONALD L. GRANGER, MOSES J. BOCKARIE, CHARLES S. MGONE, MARC C. LEVESQUE, J. BRICE WEINBERG, AND NICHOLAS M. ANSTEY

International Health Program, Infectious Diseases Division, Menzies School of Health Research, Darwin, Northern Territory, Australia; Northern Territory University, Darwin, Northern Territory, Australia; Division of Infectious Diseases, Department of Internal Medicine, University of Utah and Veterans Administration Medical Centers, Salt Lake City, Utah; Division of Hematology-Oncology, Department of Medicine, Veterans Administration and Duke University Medical Centers, Durham, North Carolina; Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea; Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea; Flinders University, Northern Territory Clinical School, Darwin, Northern Territory, Australia

Abstract. Polymorphisms in the inducible nitric oxide synthase gene (NOS2) promoter have been associated with clinical outcome from malaria. These include a CCTTT repeat ($(CCTTT)_n$) 2.5 kilobases upstream from the NOS2 transcription start site, and two single nucleotide substitutions: G→C at position -954 (G-954C), and C→T at position -1173 (C-1173T). Although hypothesized to influence NO production *in vivo*, the functional relevance of $(CCTTT)_n$ and G-954C is uncertain because disease association studies have yielded inconsistent results. This study found no association between CCTTT repeat number and levels of plasma NO metabolites or peripheral blood mononuclear cell NOS activity in a cohort of asymptomatic malaria-exposed coastal Papua New Guineans 1–60 years old. This suggests that $(CCTTT)_n$ does not independently influence NOS2 transcription *in vivo*. Neither the G-954C nor the C-1173T polymorphisms were identified in this population, indicating the variability and complexity of selection for NOS2 promoter polymorphisms in different malaria-endemic populations.

INTRODUCTION

Our group and others have demonstrated an inverse association between nitric oxide (NO) production/peripheral blood mononuclear cell (PBMC) nitric oxide synthase (NOS) expression and malarial disease severity.[1–3] In contrast to the absent or minimal PBMC inducible nitric oxide synthase gene (NOS2) expression in healthy residents of temperate climates,[4,5] basal PBMC NOS expression and activity is high in apparently healthy asymptomatic residents of tropical malaria-endemic regions.[1,2] Factors potentially influencing NOS2 transcription in these populations include age, presence of subclinical coinfections, parasite polymorphisms, and host polymorphisms in NOS2 and other regulatory cytokine genes. Four polymorphisms in the NOS2 promoter have been associated with malarial disease outcome: a CCTTT microsatellite repeat ($(CCTTT)_n$) located 2.5 kilobases upstream from the NOS2 transcription start site;[6] and single nucleotide substitutions from G→C at position -954 (G-954C),[7] from C→T at position -1173 (C-1173T),[8] and A→T at position -1659 (A-1659T).[9]

Conflicting results have been obtained from studies of the $(CCTTT)_n$ polymorphism both *in vivo* and *in vitro*. Shorter forms of the CCTTT repeat (both alleles ≤ 10 repeats) and a reduced summed repeat length were associated with fatal cerebral malaria in Gambian children,[6] whereas longer forms (either allele ≥ 15 repeats) and increased summed repeat length were associated with severe malaria in Thai adults.[10] In contrast, CCTTT repeat length was not associated with malarial disease severity or NO production in Tanzanian[11] or Gabonese children,[12] nor malarial disease severity in Kenyan children.[8] Interleukin-1β- induced NOS2 transcription in transfected colonic carcinoma cells was reported to be most effective in constructs with $(CCTTT)_{14}$.[13] Another *in vitro* study reported no differences in basal or cytokine-induced NOS2 promoter activity by constructs with deletions in the CCTTT repeat region.[14] Reports on the functional relevance of the G-954C polymorphism have also led to different conclusions. This polymorphism was over-represented in Gabonese children with mild malaria compared with those with severe malaria,[7] and was associated with an increased time to symptomatic reinfection.[12] However, the G-954C mutation was not associated with risk of cerebral malaria in Tanzanian or Gambian children.[9,11]

Since geographic variation in susceptibility to malaria and disease phenotype may be due to differences in host genetics, parasite strains, and malaria epidemiology, the functional significance of these conflicting observations is unclear. We therefore assessed the relationship of the $(CCTTT)_n$, G-954C, and C-1173T polymorphisms to *in vivo* NO production in a highly malaria-exposed coastal Papua New Guinean population. This population was expected to exhibit the high basal NO production and PBMC NOS activity that is characteristic of residents in tropical environments. This high production/activity is thought to reflect chronic stimulation of NOS2 by malaria and other subclinical infections,[1,2] and has been previously used to demonstrate higher NO production in those with disease-protective NOS2 polymorphisms.[8] We carefully selected strictly-defined asymptomatic subjects, so that basal NO production/NOS activity would not be confounded by the disease responses typically associated with temporarily skewed production of pro and/or anti-inflammatory cytokines that may regulate NOS2 transcription. We also tested *a priori* hypotheses generated from the results of the aforementioned studies in relation to basal NO production.[6,10,13]

MATERIALS AND METHODS

Informed consent was obtained from all human adult participants, and from parents or legal guardians of minors, after ethical approval to conduct the study was obtained from the Health Research Ethics Committee of the Menzies School of Health Research, Northern Territory, Australia, and the Papua New Guinea Medical Research Advisory Committee.

Asymptomatic intensely malaria-exposed children and adults were recruited from two coastal villages located approximately 20 km north of Madang, Papua New Guinea as previously described (n = 216, median age = 13 years, age range = 1–60 years, 45% male, and 70% parasitemic).[15] Volunteers were excluded from enrollment if they were febrile on any of three occasions over a 24-hour period (axillary temperature ≥ 37.5°C); had taken anti-malarial or non-steroidal anti-inflammatory medication within the past week; had clinical evidence of malaria infection within the past week (fever, chills, sweats, headache, or myalgia); had a history of infection (other than the common cold) in the past week; or had diarrhea. The Madang region is characterized by infection with all four human malaria species and there is little seasonal variation in parasitemia rates.[16] Residents are estimated to receive on average close to one infective bite per day,[17] with an increase in transmission during the wet season from October to May.[18] Parasite prevalence reaches its peak of more than 60% in the 5–9-year-old age group, which is later than that commonly observed from hyperendemic regions in Africa.[16,19] Small area variations in endemicity have been reported within a 22-km radius of Madang, with the study villages located within the high epidemiologic zone.[17] Clinical malaria epidemiology in the Madang region has been well described in children 2–15 years old and is characterized by a low incidence of severe disease, with episodic mild malaria that decreases in frequency from approximately five fever episodes per year at age 2 to one per year at age 14.[19]

Baseline plasma NO metabolites (NOx) were measured in 186 subjects after a 12-hour fast that followed a low nitrate meal, using *Aspergillus* nitrate reductase coupled with the Griess reaction.[20] The NOS activity was determined in PBMC pellet lysates from 173 subjects by measuring the molar amount of ^{14}C-arginine that was converted to citrulline per milligram of total cellular protein/hour.[4] Baseline samples from the remaining subjects were either not available or rejected if the subject was symptomatic and met the exclusion criteria, or if treatment that could potentially influence NO production had been recently administered. Parasitemia status was determined by microscopy of thick blood films on two occasions over a 24-hour period as previously described.[15]

Whole blood was spotted onto filter paper in the field, from which 3-mm disks were later punched in the laboratory and then purified using a DNA purification solution according to manufacturer's instructions (Gentra, Minneapolis, MN). The whole genome was amplified in 50-μL reactions containing 1 mM dNTPs, 6 ng of random decamers (Gigaprime Kit; Geneworks, Adelaide, Australia); and five units of HotStar *Taq* polymerase (Qiagen, Clifton Hill, Victoria, Australia) in a reaction buffer containing 67 mM Tris-HCl, 2.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, and 0.01% Tween 20. Samples were initially held at 92°C for 15 minutes to activate the HotStar *Taq* and then cycled 50 times at 92°C for one minute, 37°C for two minutes, and 55°C for four minutes (after a 10-second ramp to 55°C). The DNA product from the first-round polymerase chain reaction (PCR) was subsequently used as the template in each of the second round PCRs for genotyping the $(CCTTT)_n$,[11] G-954C,[11] and C-1173T polymorphisms,[8] and was successful in 204, 215, and 212 subjects, respectively.

Plasma NOx and NOS activity data were normalized by log transformation and analyzed according to the *a priori* and post-hoc analyses by Student's *t*-tests appropriate for variance. The relationship between plasma NOx/NOS activity and summed CCTTT repeat length was analyzed using linear regression. All statistical analyses were performed using Stata 7.0 statistical software (Stata Corp., College Station, TX) and P values < 0.05 were considered to indicate statistical significance.

RESULTS

All individuals were homozygous for the wild-type G-954 and C-1173 alleles, with the G-954C and C-1173T polymorphisms being absent from the DNA of all subjects genotyped. The $(CCTTT)_n$ alleles were bimodally distributed with peaks at 11 and 13 (Figure 1a), and the summed number of both allelic CCTTT repeats was normally distributed around a mean of 24 (SD = 2.8; Figure 1b). The geometric means for plasma NOx and PBMC NOS activity were 37 μmol/L (95% confidence interval [CI] = 34–40) and 1,020 pmol/mg (95% CI = 920–1,130), respectively. As expected, these values in asymptomatic residents of a tropical environment were markedly elevated relative to that we have previously found in fasting healthy subjects from the United States (Figure 2).[5]

Since only two subjects were homozygous for "short" $(CCTTT)_n$ alleles ≤ 10 repeats,[6] we could not statistically compare NO production in these subjects with others. The summed number of CCTTT repeats[6,10] was not significantly related to plasma NOx (R^2 = 0.005, P = 0.38) (Figure 2a) or PBMC NOS activity (R^2 = 0.002, P = 0.56) (Figure 2b). Neither measure of NO production was different in those with the $(CCTTT)_{14}$ allele[13] (n = 23; $P > 0.5$ for both comparisons) or in subjects with at least one allele of ≥ 15 repeats[10] (n = 46; $P > 0.8$ for both comparisons). In a post-hoc analysis, there was no difference in NO production in subjects in whom both alleles were ≤ 11 CCTTT repeats (i.e., equivalent to the lower bimodal peak defining shorter alleles in this population, n = 35; $P > 0.25$ for both comparisons). The potential covariates age, sex, and asymptomatic parasitemia (as previously defined[15]) were examined in multivariate linear regression models but did not significantly influence the results.

DISCUSSION

We have shown that two single nucleotide NOS2 promoter polymorphisms previously linked with malarial disease severity in Africa (the G-954C[12] and C-1173T[8]) are not present in Papua New Guinean subjects exposed to intense malaria transmission. Furthermore, we have demonstrated that the distribution of CCTTT repeat lengths differs from what has been reported previously in Africa and Thailand,[6,10,11] with an increased frequency in the Papua New Guinean population of $(CCTTT)_{11}$ and $(CCTTT)_{13}$ alleles. Most importantly, we found no association between the number of CCTTT repeats and basal NO production in a large population whose basal plasma NOx and NOS activity was in excess of that we have found previously in non-malaria-exposed controls.[5] Although our measures of plasma NOx and NOS activity potentially incorporate constitutive NO production by other NOS isoforms, NO production from these other sources is likely to be minimal in the context of the high basal PBMC NOS2 expression demonstrated in asymptomatic adults and

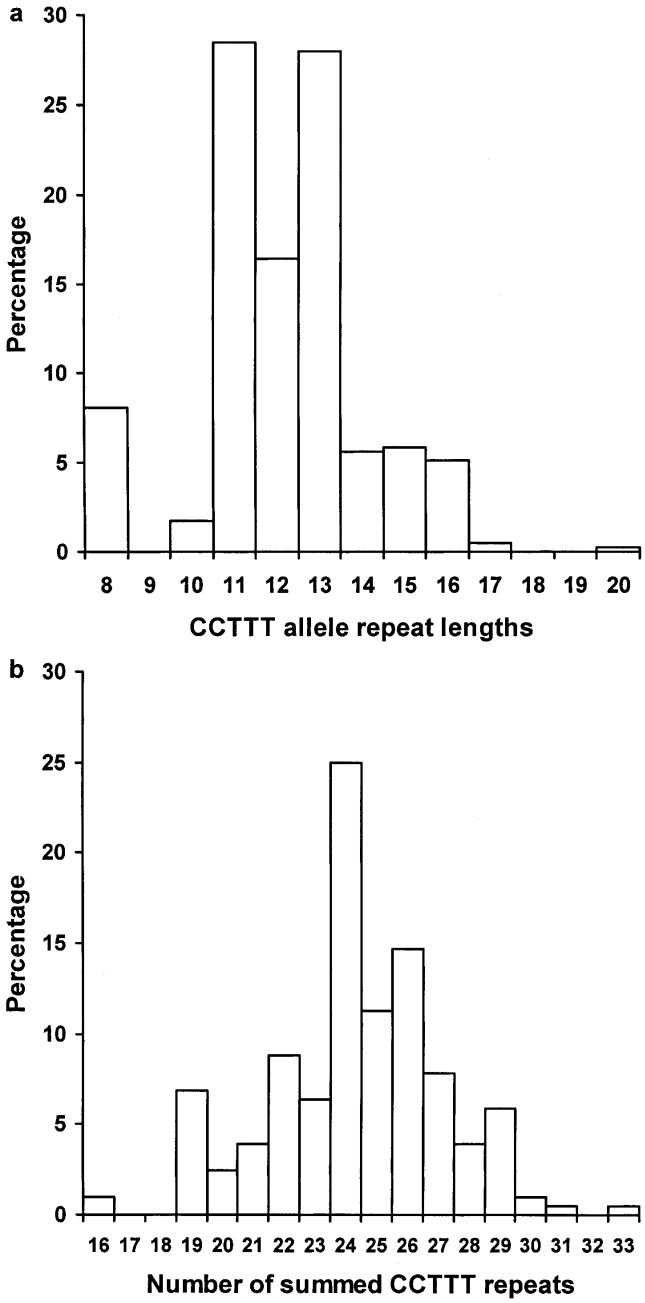

FIGURE 1. **a**, Distribution of CCTTT repeat allele lengths in the nitric oxide synthase gene and **b**, summed allele lengths of both alleles in 204 children and adults from Madang, Papua New Guinea.

children resident in tropical environments.[1,2,21] Given the contradictory nature of previous studies and the absence of functional effects in our study, it is important to reconcile our findings with those of previous reports.

If $(CCTTT)_n$ microsatellite repeat length independently influences NOS2 transcription, then we would have expected to have observed differences in chronically stimulated basal NO production *in vivo*. We have previously used such measures to demonstrate higher *in vivo* NO production in African children with a disease-protective *NOS2* polymorphism.[8] Because there was no relationship between repeat length and NO production in our study, we believe our data support the alternative hypothesis that CCTTT repeat length does not independently influence NOS2 transcription. This is further supported by the following observations: results of clinical studies are conflicting, with severe malaria associated with "short" CCTTT repeats,[6] long CCTTT repeats,[10] or not at all independently associated with $(CCTTT)_n$;[11,12] basal and cy-

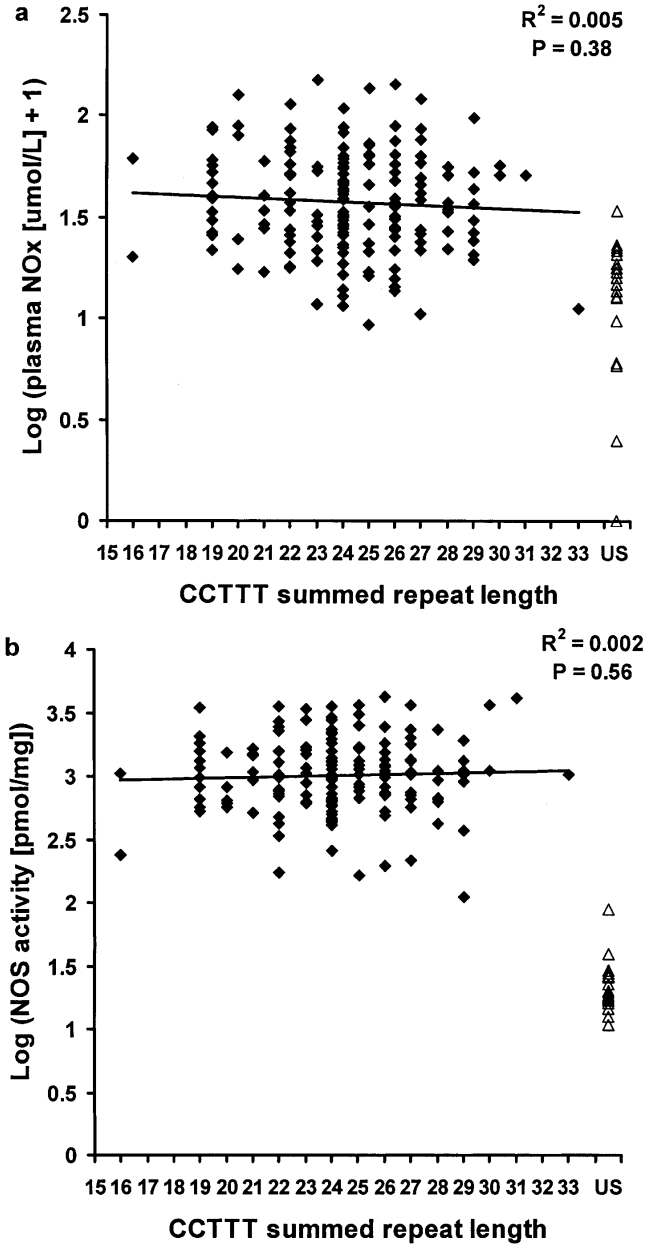

FIGURE 2. Relationship between *in vivo* nitric oxide (NO) production and summed CCTTT repeat length in the nitric oxide synthase (NOS) gene (i.e., of both alleles). **Diamonds** represent **a**, the fasting plasma NOx (n = 186) and **b**, NOS activity (n = 173) of malaria-exposed Papua New Guinean children and adults (age range = 1–60 years). The results of linear regression analyses of the data from Papua New Guinea are represented by solid lines. **Open triangles** represent comparative data from 20 healthy adult controls from the United States (age range = 31–66 years) in whom plasma NOx was measured after 24 hours on a low nitrate diet. The baseline characteristics of these 20 controls have been previously described.[5] The geometric mean plasma NOx and NOS activity were significantly higher in Papua New Guinean subjects than in controls from the United States ($P < 0.001$ for both comparisons).

tokine-induced *in vitro* NOS2 promoter activity was unaffected in luciferase constructs with deletions in the CCTTT repeat region;[14] and the number of post-hoc analyses in genetic association studies may be under-appreciated and the significance of positive associations may not withstand statistical correction for multiple comparisons.

The G-954C C-allele frequencies in malaria-endemic sub-Saharan African populations have varied from 7% to 15%[11,12] compared with only 1–4% in low-endemicity Thais[10] and in an unidentified Papua New Guinean population that included subjects with malaria,[10,12] and an absence from Germans[7] and whites in the United States.[11] The C-1173T polymorphism was present with an allele frequency of 4% in Tanzanian adults and western Kenyan children, but was absent from whites from the United States.[8] Selective pressure for these polymorphisms may be altered by variation and fluctuation in malarial transmission intensity on a geographic and temporal basis, as well as by exposure to multiple other diseases. The presence and prevalence of genetic polymorphisms may also vary among populations depending on factors other than selective pressure, including founder effect, migration, population sub-sampling, and various sociocultural factors that influence purely random mating. Thus, it is not surprising that there are major differences among populations in the genes controlling immunity to malaria, especially given the potential for polymorphisms in other genes influencing anti-malarial immune responses and the necessity for balance in adaptation to other diseases.

The G-954C polymorphism was associated with higher basal NOS activity in cultured PBMCs, but did not appear to be associated with significantly increased NOS activity in freshly collected PBMCs *ex vivo*, in which NOS activity was 10-fold higher than in the cultured cells.[12] Although associated with protection against severe anemia and hyperparasitemia in Gabon,[7] no association was found between the presence of the G-954C C-allele and either risk of cerebral malaria or NO production in Tanzanian children[11] or with risk of cerebral malaria in Gambian children.[9] The C-1173T polymorphism was associated with increased basal *in vivo* NO production and a substantially reduced risk of clinical malaria in Tanzanian children, and of severe malarial anemia in Kenyan children.[8] The absence of the G-954C and C-1173T polymorphisms from our Papua New Guinean population emphasizes that selection of particular NOS2 polymorphisms protective in African populations is not universal among highly malaria-exposed populations, and precluded assessment of functionality in this setting.

How then should the present results be interpreted? First, our results highlight the importance of subjecting hypotheses derived from post-hoc analyses to prospective analysis in follow-up functional correlation studies. Second, our data emphasize that correlation studies should be directed at uncovering whether the proposed polymorphisms (or linked "critical" polymorphisms) are present in regions that directly influence activation or repression of transcription. Third, we have identified geographic differences in the prevalence of potentially important immunoregulatory polymorphisms in populations of similarly intense malaria transmission, suggesting that other undefined NOS2 promoter polymorphisms may be functionally important in the Papua New Guinean population. Alternatively, it is possible that immune responses related to disease severity in one geographic region may be less important in others where host genetics, parasites, and malaria epidemiology may differ.

In conclusion, we have demonstrated that the $(CCTTT)_n$ microsatellite repeat is not related to chronically stimulated basal NO production in highly malaria-exposed coastal Papua New Guinean subjects. If the genetically determined capacity to produce NO is critical to anti-malarial immunity, then our results and those of previous studies linking $(CCTTT)_n$ with divergent disease outcomes in post-hoc analyses[6,10] at the very least question the universal relevance of this polymorphism. The absence in a highly malaria-exposed Papua New Guinean population of two other single nucleotide polymorphisms associated with malarial disease severity in African populations indicates the variability and complexity of selection for NOS2 promoter polymorphisms in malaria-endemic populations.

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Authors' addresses: Craig S. Boutlis, Robyn L. Marsh, and Nicholas M. Anstey, International Health Program, Infectious Diseases Division, Menzies School of Health Research, PO Box 41096, Casuarina, Darwin, Northern Territory, 0811, Australia, Telephone: 61-8-8922-8196, Fax: 61-8-8927-5187, E-mail: anstey@menzies.edu.au. Maurine R. Hobbs, Ariana N. Tkachuk, Jennifer Booth, and Donald L. Granger, Division of Infectious Diseases, Department of Internal Medicine, University of Utah and Veterans Administration Medical Centers, 30 North 1900 East, Room 4C108, Salt Lake City, UT, 84132-2405, Telephone: 801-585-1677, Fax: 801-585-3377. Mary A. Misukonis, Marc C. Levesque, and J. Brice Weinberg, Division of Hematology-Oncology, Department of Medicine, Veterans Administration and Duke University Medical Centers, 508 Fulton Road, Durham, NC, 27705, Telephone: 919-286-6833, Fax: 919-268-6891. Moses Lagog and Moses J. Bockarie, Papua New Guinea Institute of Medical Research, PO Box 378, Madang, 511, Papua New Guinea, Teelphone: 675-852-2909, Fax: 675-852-3289. Charles S. Mgone, African Malaria Network Trust, PO Box 33207, Dar es Salaam, Tanzania, Telephone: 255-22-270-0018 ext. 104, Fax: 255-22-270-0380.

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