Nitric Oxide Synthase 2^{Lambaréné} (G-954C), Increased Nitric Oxide Production, and Protection against Malaria

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A point mutation in the promoter of the nitric oxide synthase 2 gene *(NOS2),* termed NOS2^{Lambaréné} (NOS2-G954C), protects heterozygous carriers against severe malaria as effectively as the sickle cell trait. In a prospective longitudinal study, 841 individual infections of initially 200 children (151 wild-type vs. 49 NOS2^{Lambaréné} carriers) were monitored for 4 years, to assess the rates of malarial attacks in the 2 groups; carriers of the NOS2^{Lambaréné} polymorphism were significantly less likely to experience malarial attacks than were others (P = .002). The distribution of the NOS2^{Lambaréné} polymorphism was investigated in malaria-endemic areas. It was found to be present with the highest frequency in Africa and at a lower frequency in Asia. Ex vivo studies showed that cells isolated from people with this polymorphism have a 7-fold higher baseline NOS activity, compared with the levels detected in cells from subjects with the wild-type gene (P = .003).

Approximately one-third of the world's population lives in malaria-endemic regions, and ~1 million people die of malaria's consequences each year [1]. In highly endemic areas, young children are the most affected by malaria [2]. This continuous high exposure of human populations to the life-threatening parasite *Plasmodium falciparum* for >6000 years [3] has led to the hypothesis that malaria is a powerful selective force on the human genome.

One example is the sickle cell gene, which can be fatal in homozygous people if not treated appropriately before the carrier reaches reproductive age. Heterozygous carriers, however, experience relative protection against the development of severe malaria and thus promote the inheritance of the gene, despite the apparent selection disadvantage [4]. Other polymorphisms have been associated with the clinical course or occurrence of malaria as well. Examples include polymorphisms in the tumor necrosis factor promoter [5, 6], the mannose-binding lectin gene [7], major histocompatibility genes [8], and the red blood cell polymorphisms [9–13].

To provide protection against severe malaria, the effect of a mutation should influence the course of infection primarily in naive people and in those not yet frequently infected. Frequent exposure to the parasite leads to a state of semi-immunity, which provides protection against clinical complications and death but not entirely from the clinical symptoms of disease [14]. Innate immunity therefore has an exceptionally important role in preventing death from malaria by providing protection until the state of semi-immunity is reached.

Infection by a malarial parasite initiates a cytokine cascade that elicits antiparasitic properties, at least in part through the release of oxygen and nitrogen radicals. Experimental evidence shows that nitric oxide (NO) and its scavengers have an important role in antiplasmodial defense in vitro and in vivo [15–19]. In addition, elevated plasma levels of NO have been reported in patients from different endemic areas in Africa, South America, and Southeast Asia [20]. These studies suggest that high-level NO production during a bout of malaria is dependent on the severity of the disease and the degree of immunity to malaria.

NO is generated by a group of enzymes called the NO synthases (NOS) via the conversion of L-arginine to L-citrulline in the presence of various cosubstrates. The inducible NOS isoform (NOS2) represents the high-output pathway for NO production and is regulated primarily at the transcriptional level by the action of inflammatory cytokines and toxins [21]. Pharmacological inhibition of NOS2 causes a higher rate of mor-

Received 19 January 2001; revised 18 April 2001; electronically published 26 June 2001.

This study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital. Informed consent was obtained from the parents of participating children.

Financial support: *f*ortüne program from the Medical Faculty of the University of Tübingen and the European Union (Confirming the International Role of Community Research with Developing Countries).

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The Journal of Infectious Diseases 2001; 184:330–6

0022-1899/2001/18403-0011$02.00

tality in mice infected with *Plasmodium vinckei* [22], and clinical studies have demonstrated a protective role of NO in *P. falciparum* malaria, convincingly deduced from the association of NO plasma concentration with the clinical and parasitological course of the disease in individual patients [23, 24]. Taken together, these data led us to search for polymorphisms in the promoter region of the *NOS2* gene that would be associated with a geographical distribution that parallels either past or present malaria endemicity, different clinical outcomes, and physiologic changes in enzymatic activity.

In a previous report, we demonstrated that a polymorphism in the *NOS2* gene 954 nt upstream of the transcription start site (termed $NOS2^{Lambaréné}$ [in what follows, we use the more systematic term G-954C for the polymorphism]) was distributed unevenly among people with severe and mild malaria [25]. We concluded that NOS2G-954C carriers experience a certain degree of protection against the development of severe malaria by an as yet unknown mechanism. In the present report, we extend our findings by investigating (1) the geographic distribution of NOS2G-954C carriers in different populations, (2) the risk of reinfection in NOS2G-954C carriers, compared with people with the wild-type allele, and (3) the NOS enzymatic activity in cells isolated from children with the NOS2G-954C allele.

Patients and Methods

Patients and statistics. Patients from a case-control study at the Albert Schweitzer Hospital in Lambaréné, Gabon, were enrolled, as reported elsewhere [26]. In brief, children with severe malaria were included in the study when they presented with hyperparasitemia, severe anemia, or both; severe hyperparasitemia was defined as >250 asexual parasites/nL (corresponding to >10% of infected erythrocytes), and severe anemia was defined as hemoglobin level <50 g/L. Patients who were homozygous for hemoglobin S were excluded. Additional complications, such as cerebral malaria, hypoglycemia, or lactic acidosis, were recorded. For every child with severe malaria, an age-, sex-, and provenance-matched child with mild malaria was enrolled as soon as possible.

To be defined as a mild case, the following criteria had to be fulfilled: *P. falciparum* malaria with parasitemia of 1–50 parasites/nL at admission, no schizontemia, malarial pigment containing circulating leukocyte count <50 cells/μL, absence of homozygosity for hemoglobin S, hemoglobin level >80 g/L, platelet count >50 cells/nL, leukocyte count <12 cells/nL, lactate level <3 m*M,* blood glucose level >50 mg/dL, no signs of severe malaria or other acute infections, no intake of antimalarial drugs within the preceding week, and no history of hospitalization (to exclude those who already had a severe malarial attack). Routine pediatric micromethods were used to measure hematological and biochemical parameters. Giemsa-stained thick and thin blood smears were performed to quantify parasitemia [27] and for the detection of schizontemia and the quantification of hemozoin-containing neutrophils and monocytes [28].

Children with severe malaria were hospitalized and received intravenously administered quinine plus clindamycin chemotherapy for 4 days and supportive treatment, as required [29]. Children with mild malaria received a single orally administered dose of sulfadoxine-pyrimethamine [30]. After discharge from the hospital, the children were prospectively and actively followed up with visits to their homes once every 2 weeks, to obtain information about reinfection. All children who later experienced symptomatic *P. falciparum* infections (parasitemia and fever) received antimalarial chemotherapy with sulfadoxine-pyrimethamine and were recorded as having reinfections.

For the study of NOS enzymatic activity, 25 healthy children from the followed cohort were investigated. Children were given a routine physical examination, and a thick blood smear was used to verify that participants were free of parasites. Subjects were excluded from the study if they had a positive thick blood film for malarial parasites or if they had experienced malaria or any other severe illnesses within the last 4 months. The subjects previously had been genotyped for the NOS2G-954C polymorphism and were divided into 2 groups: children heterozygous for the NOS2G-954C polymorphism ($n = 14$; 8 boys and 6 girls, with a mean age of 6 years and 7 months) and children with the wild-type phenotypic expression ($n = 11$; 6 boys and 5 girls, with a mean age of 6 years and 5 months).

We used nonparametric methods for statistical testing. The Mann-Whitney *U* test was used for group comparisons for nonpaired samples, and the Wilcoxon rank sum test was used for paired comparisons. Group comparisons in the survival statistics were done with the Breslow-Gehan-Wilcoxon test. All *P* values given are 2-tailed. The level of significance was set at 2-tailed $P < .05$.

To determine the frequency of NOS2G-954C, blood samples from autochthonous inhabitants of Senegal, Nigeria, Gabon, Thailand, Papua New Guinea, and Germany were used.

Genotyping. DNA was extracted with the QIAamp blood kit (Qiagen). Polymerase chain reaction (PCR) was performed on a rapid cycler (Idaho Technologies) in 15-μL reactions. The conditions used were as follows: an initial 15-s denaturation step at 94°C, followed by 40 cycles with 0 s at 94°C, 0 s at 60°C, and 30 s at 72°C. Primers were NOS-F (5′-CATATGTATGGGAATACTGTATTTCAG-3′) and NOS-4 (5′-TCTGAACTAGTCACTTGAGG-3′). As reported elsewhere [25], the generated fragment was subjected to a 1-h digestion with the endonuclease *Bsa*I (New England Biolabs) at 50°C and subsequently was analyzed on a 3% agarose gel. To confirm the sequence of the *Bsa*I-resistant fragments, DNA sequencing was performed with dye terminator chemistry, followed by subsequent analysis on an ABI 373 sequencer (Perkin-Elmer).

Gel shift assays. Electrophoretic mobility shift assays (EMSA) were performed to determine whether a specific DNA-binding protein recognizes the polymorphic region. Synthetically derived (Interactiva), as well as PCR-generated, double-stranded oligonucleotides were used. All EMSA shown here were performed with the following fragments (boldface represents the polymorphic position −954 in the NOS2 promoter): 5′-TTGAGTTCGA**G**ACCAGCATGGACAACATGGTG-3′ (wild type) and 5′-TTGAGTTCGA**C**ACCAGCATGGACAACATGGTG-3′ (NOS2G-954C). Labeling was performed with ^{32}P-γ–adenosine triphosphate (ATP) for polynucleotide kinase reaction or fill-in of 5′ overhanging thymidines with ^{32}P-αATP with *Taq* polymerase (Qiagen). Supershift antibodies against interferon regulatory factor (IRF)–1, IRF-2, Stat-1, Stat-2, c-Jun, E2A, and phosphotyrosine were from Santa

Cruz. Anti-phosphoserine and phosphothreonine antibodies were from Sigma Chemical.

Preparation of peripheral blood mononuclear cells (PBMC) and tissue culture. Venous blood (4 mL) was drawn into EDTA-containing vials, plasma was separated, and PBMC were prepared by density gradient centrifugation, as described elsewhere [31]. All blood samples were stored under cooling conditions and were processed within 2 h after phlebotomy. Freshly isolated mononuclear cells were plated at 1.5×10^6 into 48-well tissue culture plates in 0.5 mL of Dulbecco modified Eagle medium containing 10% pooled human serum (heat-inactivated at 56°C for 30 min) or were rapidly frozen and stored at −80°C until use. For in vitro experiments, PBMC were incubated with media alone (controls), interferon (IFN)–α2b (500 U/mL; Schering-Plough), or a mixture of lipopolysaccharide (LPS; 10 ng/mL) and IFN-γ (500 U/mL; R&D Systems). Lung endothelial cells A549 and liver endothelial cells AKN1 were cultured by standard procedures.

Measurement of NOS enzyme activity. NOS enzyme activity was analyzed by measuring the conversion of ^{14}C-L-arginine to ^{14}C-L-citrulline (NEN) in cell extracts prepared from cultured cells that were scraped and frozen and from frozen PBMC pellets; cell lysates were prepared by 5 freeze-thaw cycles in a PBS–protease inhibitor cocktail. Lysates were collected by centrifugation and were analyzed for protein concentration and NOS enzyme activity. L-arginine labeled with ^{14}C in the guanido position was added to the lysates. The conversion of L-arginine to L-citrulline was determined by the lack of adherence of L-citrulline to a column containing Dowex AG 50W-X8 cation exchange resin (Sigma). Samples were analyzed in triplicate and were expressed as picomoles of citrulline per milligram of protein. The inter- and intra-assay variation of this assay is well below 5%.

Results

In our previous study, searching for new polymorphisms that might influence the outcome of malaria, we identified a single nucleotide polymorphism −954 bases upstream of the transcriptional start site of the *NOS2* gene (NOS2G-954C). This polymorphism was significantly more frequent in patients with mild malaria ($n = 100$), compared with their matched severe malaria control subjects ($n = 100$), independently of their sickle cell genotype ($P = .040$). There was also a difference between the length of time to first reinfection between the groups: we observed 19 weeks versus 30 weeks in the wild-type and NOS2G-954C patients, respectively ($P = .022$) [25]. We concluded that this polymorphism might be involved in protection against malaria.

Next, we further investigated whether the mutation had an impact on the time to reinfection after curative treatment. In Gabon, where this polymorphism was originally identified, a longitudinal follow-up of the initial 200 children has been ongoing for several years. Forty-nine children had the NOS2G-954C mutation; the only 2 children homozygous for NOS2G-954C were among the few who moved out of the area shortly after admission and therefore could not be included in the study. Figure 1 shows that, after 4 years of follow-up, NOS2G-954C carriers show a highly significantly longer time to symptomatic reinfection, compared with the children with the wild-type gene ($P = .002$). That was not the case for sickle cell gene carriers or for other hemoglobinopathies in this location [12].

To determine whether the distribution of the NOS2G-954C mutation parallels current and past malaria endemicity, we genotyped blood samples from donors of different parts of the world by applying the same rationale that was used to establish the hypothesis that the sickle cell gene provides protection against malaria [4]. We did not find any NOS2G-954C carriers in 100 white German blood donors. Samples from Central and West Africa showed a high gene frequency (7%–10%). In Southeast Asia, the mutation was also present, but at a lower frequency (2%–4%; table 1). A relatively high frequency of the sickle cell gene is also found in sub-Saharan Africa. Lower frequencies of the sickle cell trait can also be observed in Asia and the Mediterranean basin [32].

The restricted worldwide distribution, the different frequency in mild versus severe malaria cases, and the clinical finding of a prolonged time to reinfection are evidence for a potential role of the NOS2G-954C mutation in innate immunity against malaria; these findings prompted us to look for a physiologic explanation of the epidemiological and clinical findings.

To determine whether the NOS2G-954C promoter polymorphism is associated with altered NO production, we took advantage of a method that measures NOS activity with high

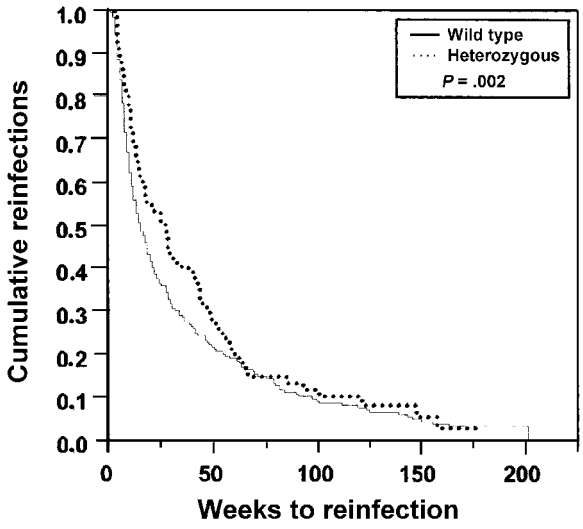

Figure 1. Kaplan-Meier plots for reinfection. Cumulative reinfections are plotted against weeks to the next infection. In total, 841 reinfections were analyzed and split by NOS2G-954C heterozygous and wild-type people. Patients from a case-control study at the Albert Schweitzer Hospital in Lambaréné, Gabon, were enrolled, as reported elsewhere [26]. The patients were actively followed-up with visits once every 2 weeks at their homes, to obtain information about reinfection.

Table 1. Gene frequencies from different regions with the NOS2G-954C mutation.

Site	Gene frequency	No. of patients
Gabon[a]	.098	193
Nigeria[b]	.068	96
Senegal[b]	.089	242
Thailand[c]	.025	59
Papua New Guinea[d]	.036	70
Germany[e]	0	100

[a] Samples from healthy schoolchildren.
[b] Samples from people presenting without parasitemia, with asymptomatic parasite infection, or with mild malaria.
[c] Samples from patients with *Plasmodium vivax* malaria.
[d] Samples from patients with mild malaria or with asymptomatic plasmodial infections.
[e] Samples from white blood donors.

sensitivity and specificity [33]. NOS enzyme activity in cultured PBMC from healthy children with the NOS2G-954C promoter polymorphism ($n = 14$) was compared with that of PBMC from children with the wild-type genotype ($n = 11$). Freshly isolated cells incubated with media alone (controls), IFN-α2b, and a combination of LPS and IFN-γ were investigated. People who were heterozygous for NOS2G-954C had a significantly higher NOS activity under baseline conditions, compared with the wild-type group (mean, 38 ± 11 p*M* in the mutant group vs. 8 ± 2 p*M* in the wild-type group; $P = .003$; figure 2*A*). Relative to control conditions, stimulation of cultures with IFN-α significantly increased NOS enzyme activity in the wild-type group (23 ± 10 p*M;* $P = .021$) but just not significantly in the NOS2G-954C group (figure 2*A*). NOS activity in the NOS2G-954C group was higher under those conditions, although just not significantly.

Treatment of cells with a combination of LPS and IFN-γ did not alter NOS activity significantly in the NOS2G-954C group but did cause a significant increase in NOS activity in the wild-type group (16 ± 4 p*M;* $P = .013$; figure 2*A*). However, relative to the wild-type group, NOS enzyme activity was higher in the NOS2G-954C group ($P = .051$; figure 2*A*). NOS enzyme activity also was evaluated in noncultured PBMC from the 2 groups of children, to determine whether the NOS2G-954C promoter polymorphism was associated with elevated ex vivo activity. As shown in figure 2*B,* NOS activity in noncultured PBMC was higher in the NOS2G-954C group, compared with the wild-type group. These findings suggest that NOS2G-954C is associated with elevated baseline NO production in vivo. Overall, we found a 10-fold higher NOS activity ex vivo than in vitro. This is a typical finding for cultured cells; they typically have much lower NOS activity (7–11-fold). This is likely because in vitro culture does not properly mimic the true in vivo environment. This could be due to any number of reasons, such as adherence or removal of a critical stimulatory factor.

We postulated that this high baseline-level activity might be due to modified binding of a constitutive acting DNA binding protein to the polymorphic site. We performed EMSA with different probes containing the polymorphic site, to determine whether DNA binding proteins specifically recognized the region. All fragments used showed retarded mobility, and this binding was specific for the fragment. Protein extracts from A549 cells produced a doublet, compared with the probe without any added protein (figure 3*A, lane 1*). AKN1 cells produced 3 bands (figure 3*B, lane O*). Competition with nonlabeled mutant and wild-type oligonucleotide showed a difference in affinity for the respective oligonucleotides. The wild type bound with lower affinity, compared with the NOS2G-954C mutant (figure 3*B*). The addition of a 64-fold molar excess of the wild-type oligonucleotide did not resolve the cDNA-protein complex

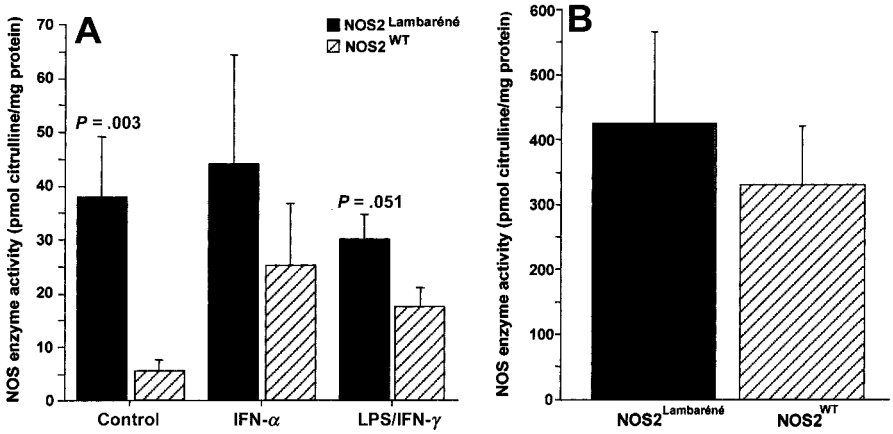

Figure 2. Nitric oxide synthase (NOS) enzyme activity in peripheral blood mononuclear cells (PBMC). *A,* PBMC from healthy children, cultured in the presence of medium alone (controls), interferon (IFN)–α2b (500 U/mL), lipopolysaccharide (LPS; 10 ng/mL), and IFN-γ (500 U/mL). *B,* NOS enzyme activity in PBMC immediately after separation from whole blood. Data are mean ± SE. WT, wild type.

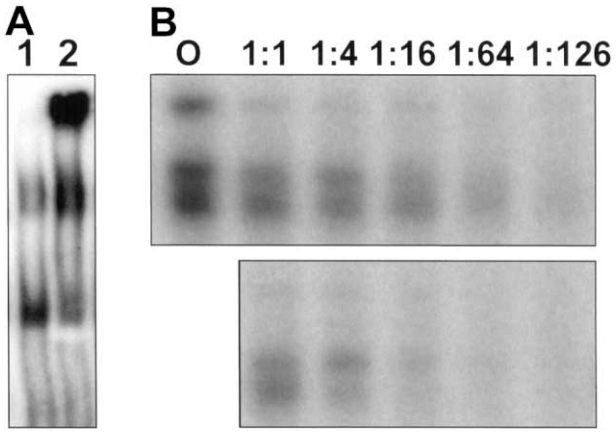

Figure 3. *A,* Electrophoretic mobility shift assay (EMSA) with a radiolabeled 30-bp oligonucleotide derived from the nitric oxide synthase 2 (NOS2) promoter. *Lane 1,* Radiolabeled oligonucleotide plus nuclear proteins; *lane 2,* radiolabeled oligonucleotide plus nuclear protein plus antiphosphoserine antibody. *B,* Competitive EMSAs with a radiolabeled NOS2 wild-type (wt) oligonucleotide and an unlabeled NOS2G-954C oligonucleotide. The autoradiograph shows 2-ng radiolabeled NOS2 wt oligonucleotide that was incubated with 10 ng of nuclear proteins from AKN1 cells. Next, unlabeled oligonucleotides were added into the assay in a ratio, as indicated above the lanes (*top,* wt oligonucleotide; *bottom,* NOS2G-954C oligonucleotide).

completely (figure 3*B, top, lane 1:64*). In contrast, when the same molar excess of the mutant oligonucleotide was added, no DNA/protein complex could be observed (figure 3*B, bottom, lane 1:64*). This was consistently shown in all possible competition combinations.

To obtain information about possible modifications of the binding protein, we performed supershift experiments. Antibodies derived against phosphorylated serine caused a strong retardation (figure 3*A, lane 2*), whereas other antibodies against phosphorylated threonine or tyrosine did not cause a mobility change (data not shown). There is no obvious DNA target in the databases that could unveil the identity of the possible binding factor on the basis of the DNA sequence of the binding site.

Supershift experiments with antibodies against IRF-1, IRF-2, Stat-1, Stat-2, c-Jun, and E2A did not give any hint toward the identity of the DNA binding protein or proteins (data not shown). Formation of the DNA-protein complex was found with nuclear extracts from all epithelial cell lines (A549, AKN-1, and HUH-7), as well as in cells of the hematopoietic lineage, such as JY B cells and U937 cells. However, we detected no complex formation in Jurkat T cells.

Discussion

Most other studies investigating genetic polymorphisms in malaria rely on very large sample sizes in cross-sectional studies, at the cost of a less defined study cohort. Our aim was to combine an intense selection of the patients at admission with a close follow-up of the relatively large sample, to obtain insights in possible mechanisms of the pathogeneses that influence the course of malaria on a given genetic background. To control the efficacy of our approach, we typed our patients for the sickle cell gene. As expected, we found a significantly higher frequency of sickle gene carriers in the group with mild malaria [25]. Because sickle cell trait is a genetic factor known to provide relative protection against the development of severe malaria [32], we concluded that our grouping and study size were adequate to detect genetic polymorphisms that have relatively strong effects on malaria severity [12, 25].

An interesting finding was that patients with an initial severe malaria episode continued to have an increased risk of developing severe malaria in the follow-up phase. This likely reflects an inborn susceptibility to develop severe malaria. Socioeconomic and cultural differences do not seem to explain the differences in the cohort investigated [34]. Preliminary results also exclude entomological factors as influential sources for disease outcome and reinfection rate [35].

One important difference between our study and others is that the number of cerebral malaria cases is quite low. Therefore, mutations described to be associated with susceptibility to cerebral malaria are not found in our patient grouping. On the other hand, geographic differences in parasitological or ethnic parameters could influence the level of resistance; a study in Tanzania detected no differences in NOS2 activity between the wild-type and NOS2G-954C populations [36]. It has to be stated, however, that the experiments were designed differently, and the study in Tanzania was only cross-sectional. Also, in other studies where no differences were found, usually NO metabolites in plasma, urine, or both were measured. These measurements are a reflection of whole-body NO production and are highly influenced by diet. For these reasons, we measured NOS enzyme activity in PBMC. Our measurements are thus not influenced by diet, are more sensitive, and are more quantifiable because they are a direct measure of cell-specific production of NO.

We also investigated the length polymorphism in the pentanucleotide repeats upstream of the *NOS2* gene. A recent study associated this polymorphism with susceptibility to cerebral malaria [37]. We could not detect any significant effect of this polymorphism in our study (data not shown). Also, the tumor necrosis factor promoter mutation –108 was not distributed differently between the groups (data not shown).

Here, we describe a single nucleotide polymorphism in the promoter region of the NOS2 promoter (NOS2G-954C) that geographically mirrors present endemicity. It also is associated with protection against malaria in African children. Clinically, we find significantly longer times between malarial attacks in children with the NOS2G-954C polymorphism. This is, to our knowledge, the first gene mutation investigated in a longitudinal study describing malarial reinfections that shows a protective effect during a 4-year period of intense follow-up.

On the physiological level, we found a DNA binding protein that has a different affinity to the polymorphic site and an increased in vitro and ex vivo NO production in children with the mutation. These findings led us to hypothesize that the NOS2G-954C mutation is protective in nonimmune people via the constitutively high baseline level of NOS activity. The protein that binds to the region where the promoter mutation is found could be an activator of gene expression. Because it binds with a higher affinity to the mutant site than to the wild-type sequence, a higher base level of NOS activity can be found.

Two locations of action for the killing of parasites by NO are possible. Parasites that invaded the liver might provoke a cytokine response (e.g., IFN-γ). In return, this response induces NOS2, which will produce NO and subsequently kill the invading parasite. In people who carry the NOS2G-954C polymorphism, the constitutively high level of NOS activity in heterozygous people are at an advantage in resisting the liver stages of the parasites without the need of an induction by other stimuli. An alternative hypothesis is the involvement of NO in reducing blood-stage parasite density by an antibody-dependent cellular inhibition of parasites type of effector mechanism. The antibody-dependent cellular inhibition is triggered by ingestion of opsonized merozoites by activated monocytes that results in the release of toxic products acting in trans on maturing intracellular parasites. Because activated macrophages are adhering cells, one attractive possibility is that adherent activated monocytes-macrophages may release NO locally after ingestion of merozoites. This released NO could then kill cytoadherent parasites located in their vicinity. Such a mechanism is predicted to prevent occurrence of high parasite density—that is, one of the phenotypes observed in our field studies.

Acknowledgments

We thank the technical staff at the study locations, particularly Andrea Weierich and Mary Misukonis for excellent technical help. We also thank the members of the 1/95C study team: B. Bojowald, O. Dangelmaier, B. Greve, K. Herbich, H. Knoop, L. Lehman, B. Lell, D. Luckner, P. Matousek, A. Missinou, S. Pabisch, P. Rezbach, D. Schmid, R. Schmidt-Ott, and M. Sovric.

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