Am. J. Trop. Med. Hyg., 65(5), 2001, pp. 639–643

ASSOCIATION OF SPLENOMEGALY WITH CEREBRAL MALARIA AND DECREASED CONCENTRATIONS OF REACTIVE NITROGEN INTERMEDIATES IN THAILAND

MATHIEU NACHER, PRATAP SINGHASIVANON, SOMBAT TREEPRASERTSUK, YUPA CHANTACHUM, SUPARP VANNAPHAN, BOUBACAR TRAORE, FRÉDÉRICK GAY, AND SORNCHAI LOOAREESUWAN

Institut National de la Santé et de la Recherche Médicale Unité 511, Immunobiologie Cellulaire et Moléculaire des Infections Parasitaires, Faculté de Médecine Pitié-Salpétrière, Paris, France; Faculty of Tropical Medicine, Mahidol University, Hospital for Tropical Diseases, Bangkok Thailand

Abstract. The role of the spleen during *Plasmodium falciparum* malaria in humans is unclear. In Thailand, malaria transmission is low and splenomegaly is rarer than in high transmission areas. We compared the prevalence of splenomegaly between 52 cerebral malaria patients and 191 patients without complications despite a high parasite biomass. We also measured concentrations of reactive nitrogen intermediates (RNIs) in a fraction of these cases recruited in 1998 (24 cerebral malaria and 56 controls). Splenomegaly was significantly associated with cerebral malaria (adjusted odds ratio = 2.07 [95% confidence interval = 1–4.2]; $P = 0.048$). There was a linear trend for this association ($P = 0.0003$). After adjusting for potential confounders, concentrations of RNIs were significantly lower in the presence of splenomegaly ($P = 0.01$). These results suggest that in humans, as in animal models, the spleen may be involved in the pathogenesis of cerebral malaria. The relationship between RNI concentrations and the spleen suggest that nitric oxide may have a regulating role in the complex physiology of the spleen during malaria.

INTRODUCTION

The role of the spleen in malaria is thought to be of major importance in the destruction of parasitized erythrocytes. In animal models, before crisis occurs, there is a marked reduction in the cordal microcirculation shunting the spleen from the general circulation, which reduces splenic trapping of abnormal erythrocytes.[1] During crisis, the splenic circulation is restored, leading to a massive parasite destruction within the spleen. Blockage of cordal circulation by cellular hyperplasia resulting from immunologic stimulation and accumulation of erythroid precursors have been proposed to explain these functional changes. During crisis, the release of reticulocytes and possibly microvascular mechanisms will restore efficient splenic trapping of infected erythrocytes. The concomitant massive release of immune effectors leads to parasite destruction.

In humans, the role of the spleen is still unclear and few studies have directly addressed the problem. Studies on human splenic function in Thailand during mild *Plasmodium falciparum* malaria showed an increase of splenic clearance in patients with splenomegaly,[2] and suggested that clearance was even greater in severe malaria.[3] A clinical study in Zambia, comparing the prevalence of splenomegaly during cerebral malaria and mild malaria, suggested a protective role for the spleen.[4] Cerebral malaria patients with splenomegaly had a better prognosis than those without splenomegaly.[5] However, the prevalence of splenomegaly during cerebral malaria varied widely.[6,7] These studies used different comparison groups and were conducted in areas of different transmission patterns. In Thailand, transmission is low and splenomegaly is much rarer in exposed populations.[8,9] Therefore, comparing the prevalence of splenomegaly between cerebral malaria and non-severe malaria in this population may yield results differing from areas of high transmission.

In murine models, nitric oxide (NO), a major immune effector, has the ability to suppress the stimulation of Th1 lymphocytes, and the release of interleukin-2, tumor necrosis factor-α, and interferon-γ,[10] which are correlated with splenomegaly.[11] It also has a vascular effect on spleen circulation.[12] Because NO has a very short life, we can only rely on the measurement of its metabolites, reactive nitrogen intermediates (RNIs). Our objectives in this study were to determine if there was any significant association between splenomegaly and cerebral malaria and to compare RNI concentrations between patients with and without splenomegaly.

PATIENTS, MATERIALS, AND METHODS

This retrospective study took place between 1998 and 1999 at the Hospital for Tropical Diseases in Bangkok, Thailand. Transmission of malaria in endemic areas of Thailand is seasonal with very low entomologic inoculation rates ($<$ 1 infected bite per person per year). Fifty-two consecutive cerebral malaria patients were compared with 191 consecutive patients with a high parasite biomass and 371 controls with mild malaria. On a subgroup recruited consecutively during 1998 (24 cerebral malaria cases and 56 patients without cerebral malaria despite a high parasite biomass), we collected a 5-ml blood sample, from which serum was frozen at -40°C for subsequent measurements. All patients gave informed consent before blood was drawn, and the study was approved by The Ethical Committee of Mahidol University. Mixed infections and hemoglobinopathies were excluded, since they are associated with splenomegaly.

Definitions. Cerebral malaria was defined by a Glasgow Coma Score $<$ 10. Non-severe malaria with high parasite biomass[13] was defined as a parasitemia $>$ 5% of red blood cells or more than 200 000 parasites/μl and presence of circulating schizonts on a blood smear and absence of any severity criteria.

Mild malaria was defined by presence of *P. falciparum* on the blood slide and less than 5% of parasitized erythrocytes or 200 000 parasites/μl and absence of circulating schizonts on a blood smear.

Variables. The Hospital for Tropical Diseases conducts numerous clinical trials; therefore, patient files contain standardized information, including spleen size and the duration of the illness. Spleen size is determined by palpation on ad-

TABLE 1
Frequency of splenomegaly in different groups of patients with *Plasmodium falciparum* malaria in Thailand

	Cerebral malaria	High parasite biomass but non-severe	Mild malaria
Mean age (± SD), years	29 (± 10)	26 (± 11)	25 (± 10)
Median symptoms duration (days) [interquartile range]	5 [4–9]	4 [3–6]	4 [2–7]
Geometric mean parasitemia (trophozoites/μl)	41,333	97,600	12,700
Geometric mean peripheral schizont count (schizonts/μl)	61	35	0
Splenomegaly (%)			
No	29 (56)	145 (76)	284 (77)
Yes	23 (44)	46 (24)	87 (23)
Staging of splenomegaly (%)			
1	5 (9)	23 (12)	47 (13)
2	8 (15)	14 (7)	24 (6)
3	10 (19)	9 (5)	15 (4)

mission by one of the senior physicians. Size is expressed in fingerbreadths below the rib line.

We reviewed case record forms from 1998 to 1999. To avoid single observations and to have comparable spleen size categories, we grouped patients with a splenomegaly of three or more finger breaths below the rib line in one category. This did not modify the dose effect trend. We used spleen size both as a semi-quantitative variable and as a qualitative variable presence/absence. For all these patients the evolution duration of symptoms was recorded to adjust for the effect of time and thus have patients with comparable parasite growth kinetics. Patients had full clinical examination and routine biological examinations. Concentrations of RNIs were obtained using spectrophotometry and the Griess modified technique.[14] We also measured total IgE and sCD23 concentrations. Because of insufficient volume of plasma aliquots and the numerous measurements performed, the number of complete observations was lower for CD23 and IgE than for RNI measurements. The final volume of serum per aliquot, was not to our knowledge, related to the studied variables; notably, it was not related to the blood pressure on admission. Therefore, and given the level of statistical significance, we are confident it did not affect the validity of our results.

Comparison groups. It has been suggested that at a population level, the relative rarity of cerebral malaria reflects that of the most virulent strains.[15] Thus, if parasite factors are a major determinant in the pathogenesis of cerebral malaria, unidentified strain differences would confound the ability to measure the effect of host factors influencing susceptibility to cerebral malaria when the study relies upon comparing mild malaria with cerebral malaria. Comparing a majority of patients infected by a virulent strain with a minority of patients infected by a virulent strain could at times seem like comparing patients infected by different pathogens. The best control group would be patients infected by the same strain who did not develop cerebral malaria. In the absence of known stable virulence markers, and since there is a positive correlation between parasite biomass and complications,[14] we chose controls with a high parasite biomass but no severity defining criteria and adjusted for the symptoms' duration. Patients thus had parasites of comparable growth kinetics, and only differed from the clinical status reflecting presence or absence of cerebral dysfunction. Thus, after frequency matching for parasite biomass and adjusting for time we assume this would reduce the specific effect of the strain, and allow cases and controls to be submitted to comparable baseline experience in terms of the parasite determinant, therefore facilitating and unbiasing the detection of host-related factors. We also included mild malaria cases (*P. falciparum* infection without any severity criteria and without hyperparasitemia, nor peripheral schizonts) as a 'classical' control group. Another advantage of using the high biomass controls was that it reduced the referral hospital bias: the Hospital for Tropical Diseases is a referral hospital for severe malaria (broad definition including hyperparasitemia); therefore, both severe malaria *stricto sensu* and hyperparasitemia were admitted through similar channels, whereas patients with mild malaria were often admitted in this hospital for socioeconomic reasons. A study on background data showed that mild malaria often had a lower socioeconomic status had been living longer in the area were they contracted malaria and were more likely to have a history of malaria than patients with severe malaria and patients with a high parasite biomass.

Statistical methods. Qualitative variables yielded crude odds ratios. We adjusted for potential confounders using logistic regression models forcing adjustment variables in the model. The semi-quantitative staging of splenomegaly allowed us to obtain a linear trend chi-square. The RNI concentrations were compared using Student's unpaired *t*-test. We subsequently adjusted for potential confounders using multiple linear regression. To explain the variance of RNI concentrations in different groups we used multiple linear regression models. The best model was chosen according to its adjusted R^2 value.

RESULTS

Splenomegaly and cerebral malaria. The frequencies of splenomegaly per group are shown in Table 1. When cerebral malaria and non-severe malaria with a high parasite biomass were compared, splenomegaly was significantly associated with cerebral malaria (Table 2). Adjustments on evolution duration, age, schizontemia, and parasitemia did not alter this association (Table 2). There was a linear trend for this association (Table 2). When cerebral malaria and mild controls were compared, this difference was also present. There was no correlation between symptoms duration and splenomegaly (Spearman's $\rho = 0.07$, $P = 0.13$) or between

TABLE 2
Odds ratios and linear trend for splenomegaly when comparing cerebral malaria with different control groups

	Cerebral malaria compared with high parasite biomass but non-severe malaria	Cerebral malaria compared with mild malaria
Splenomegaly (present/absent)		
Crude odds ratio	2.32	2.3
(confidence interval)	(1.24–4.36)	(1.26–4.3)
P	0.004	0.007
Splenomegaly (present/absent)		
Adjusted odds ratio*†	2.07	2
(confidence interval)	(1–4.2)	(1–4)
P	0.048	0.047
Adjusted odds ratio*†		
(using splenomegaly as a semi-quantitative variable)	1.56	1.5
[confidence interval]	[1.1–2.2]	[1.1–2.04]
P	0.008	0.01
Staging of splenomegaly		
Odds (confidence interval)		
0	0.2 (0.15–0.32)	0.09 (0.06–0.14)
1	0.22 (0.08–0.57)	0.1 (0.04–0.25)
2	0.57 (0.24–1.36)	0.29 (0.13–0.67)
3	1.1 (0.45–2.7)	0.5 (0.28–1.37)
Trend χ^2 (1 degree of freedom)	13.31	21
P	0.0003	0.0001

* Adjustment for age, symptoms duration, schizont counts, and parasitemia using unconditional logistic regression.
† Adjustment for age, symptoms duration, and parasitemia using unconditional logistic regression.

parasitemia and splenomegaly (Spearman's $\rho = -0.04$, $P = 0.3$).

Splenomegaly and RNIs. The mean ± SD optic density of reactive nitrogen derivatives was higher in patients without splenomegaly (68 ± 22, n = 52) compared with those with splenomegaly (60 ± 16, n = 28; $P = 0.09$). Adjusting for parasitemia, schizont counts, creatininemia, clinical status (cerebral malaria, controls), and evolution duration showed that RNI concentrations were significantly lower when the patient had splenomegaly ($P = 0.01$).

Different multiple linear regression models predicting RNI concentrations among cerebral malaria cases and controls with a high parasite biomass also showed that RNI concentrations were lower in the presence of splenomegaly (Table 3). When splenomegaly was used as a semi-quantitative variable, there was a negative correlation between RNI and splenomegaly in cerebral malaria cases (Spearman's $\rho = -0.35$, $P = 0.06$) but not in controls with a high parasite biomass (Spearman's $\rho = -0.1$, $P = 0.3$).

TABLE 3
Multiple linear regression models explaining the variance of reactive nitrogen intermediate (RNI) concentrations in cerebral malaria and in controls with no complications despite a high parasite biomass

	Coefficient for splenomegaly (*P*)	Adjusted r^2 (P)
Cerebral malaria* (available observations n = 20)	−10.7 (0.001)	0.48 (0.01)
Non-severe malaria with high parasite biomass† (available observations n = 37)	−14 (0.009)	0.58 (< 0.0001)

* Best model predicting 48% of the variance of RNI concentrations includes parasitemia, schizont counts, body mass index, and creatininemia using multiple linear regression analysis.
† Best model predicting 58% of the variance of RNI concentrations includes log total IgE, log sCD23, and leukocyte counts.

RNI and parasitemia. There was no significant difference in RNI optical density between cerebral malaria cases and controls with high parasite biomass (71 ± 20, n = 24 versus 64 ± 21, n = 56; $P = 0.14$). After adjustments for parasitemia, schizont counts, and creatininemia, this remained insignificant ($P = 0.12$). There was no significant correlation between symptoms duration and RNI (Spearman's $\rho = -0.13$, $P = 0.2$).

DISCUSSION

The association of splenomegaly with cerebral malaria is in contrast with the results of other studies suggesting that splenomegaly is associated with protection.[4,5] In Thailand malaria transmission is low and infections are usually symptomatic. The prevalence of splenomegaly in patients is also low and patients may develop cerebral malaria at all ages.[8,9] In areas of high transmission, the prevalence of splenomegaly is much higher, especially in children as they acquire specific immunity. Therefore, it is possible that the association of splenomegaly with protection was confounded by the background immunity or other causes of splenomegaly influencing the risk of developing cerebral malaria such as hemoglobinopathies or parasitic diseases. It is also possible that chronic and acute spleen stimulation have different effects on the response of the spleen. Since our study was a case records study, it is possible to conceive that patients with non-severe malaria were examined less thoroughly than patients with severe malaria; thus splenomegaly was missed in these patients. However, we do not believe this was the case because the odds ratio was similar when using controls with no severity definition criteria despite a high parasite biomass, who are always admitted into the intensive care unit for close monitoring. Therefore, the care they receive is

comparable to that received by cerebral malaria cases. The association between cerebral malaria and splenomegaly could have simply reflected a greater stress on the immune system in patients with severe disease. However, it is possible to question a causal relationship between splenomegaly and cerebral malaria. During malaria in Thailand, there is a positive correlation between spleen size and function.[2] The presence of different animal models showing that presence of a functional spleen was necessary to develop cerebral malaria[16,17] and the linear trend between splenomegaly and cerebral malaria could support a causal relationship. Other models of malaria have also shown that during crisis the opening of the blood-spleen barrier led to parasite elimination whereas splenectomized animals died of overwhelming parasite burdens.[18,19] Therefore, the limit between a pathogenic and a beneficial function for the spleen is not clear.[20] Perhaps the timing of the blood-spleen barrier opening is of importance, with a premature opening selecting cytoadhesive clones before the immune response is fully operational.

We did not observe any significant difference between RNI concentrations in those with cerebral malaria and controls. However, RNI variations seemed related to different parameters in cases and controls with a high parasite biomass. This will be detailed in another paper. The common factor between cases and controls was the fact that RNI concentrations were significantly lower in the presence of splenomegaly. A possible explanation would be that NO release, by reducing levels of IL-2, IFN-γ, and TNF-α,[10] reduced the immune proliferation within the spleen, thus its size. The fact that there were significant differences in the prevalence of splenomegaly, but not for RNI concentrations, between cases and controls can be reconciled with the fact that in both groups RNI concentrations were lower in patients with splenomegaly (Table 3), if we assume that the NO induction pathway also affected spleen size. Th1 cytokines, may have increased immune proliferation within the spleen[10] and induced NO release, whereas Th2 cytokines, through the CD23/NO pathway,[21] may have led to NO release without prior stimulation of spleen cells.

It is also possible that the vasoactive action of NO shunted the spleen from the general circulation. Thus, NO could participate in the physiology of the blood spleen barrier during malaria and avoid selection of cytoadhesive strains. However, another possibility would be that a third factor confounded the relation between splenomegaly and RNI concentrations. To answer some of these questions, we are now conducting a study with direct splenic function measurements.

Acknowledgments: We thank the ICU staff and the Hematology Laboratory staff at the Hospital for Tropical Diseases, Bangkok for their invaluable assistance.

Financial support: This study was supported by the Bureau Régional and the Scientific Cooperation Department at the French Embassy in Bangkok, and the U.S. National Institute of Allergy and Infectious Diseases Grant A1 35827/CFDA 93856.

Authors' addresses: Mathieu Nacher, Boubacar Traore, and Frédérick Gay, Institut National de la Santé et de la Recherche Médicale Unité 511, Immunobiologie Cellulaire et Moléculaire des Infections Parasitaires, Faculté de Médecine Pitié-Salpétrière, 91 Boulevard de l'Hôpital, 75634 Paris Cedex 13, France. Pratap Singhasivanon, Sombat Treeprasertsuk, Yupa Chantachum, Suparp Vannaphan, and Sornchai Looareesuwan, Faculty of Tropical Medicine, Mahidol University, Hospital for Tropical Diseases, 420/6 Rajvithi Road, 10400 Bangkok, Thailand.

Reprint requests: Mathieu Nacher, Faculty of Tropical Medicine, Mahidol University, Hospital for Tropical Diseases, 420/6 Rajvithi Road, 10400 Bangkok, Thailand. e-mail: m_nacher@lycos.com

REFERENCES

1. Wyler DJ, Quinn TC, Chen L, 1980. Relationship of alterations in splenic clearance function and microcirculation to host defense in acute rodent malaria. *J Clin Invest 67:* 1400–1404.
2. Looareesuwan S, Ho M, Watanagoon Y, Warrell DA, Bunag D, Harinasuta T, Wyler DJ, 1987. Dynamic alterations in splenic function during acute falciparum malaria. *N Engl J Med 317:* 675–679.
3. Looareesuwan S, Davis TM, Pukrittayakamee S, Supanarond W, Desakorn V, Silamut K, Krishna S, Boonamrung S, White NJ, 1991. Erythrocyte survival in severe falciparum malaria. *Acta Trop 48:* 263–270.
4. Olweny CL, Chauhan SS, Simooya OO, Bulsara MK, Njelesani EK, Van Thuc H, 1986. Adult cerebral malaria in Zambia: preliminary report of clinical findings and treatment response. *J Trop Med Hyg 89:* 123–129.
5. Thuma PE, Mabeza GF, Biemba G, Bhat GJ, McLaren CE, Moyo VM, Zulu S, Khumalo H, Mabeza P, M'Hango A, Parry D, Poltera AA, Brittenham GM, Gordeuk VR, 1998. Effect of iron chelation therapy on mortality in Zambian children with cerebral malaria. *Trans R Soc Trop Med Hyg 92:* 214–218.
6. Waiz A, Chakraborty B, 1990. Cerebral malaria: an analysis of 55 cases. *Bangladesh Med Res Council Bull 16:* 46–51.
7. Steele RW, Baffoe-Bonnie B, 1995. Cerebral malaria in children. *Pediatr Infect Dis J 14:* 281–285.
8. Luxemburger C, Ricci F, Nosten F, Raimond D, Bathet S, White NJ, 1997. The epidemiology of severe malaria in an area of low transmission in Thailand. *Trans R Soc Trop Med Hyg 91:* 256–262
9. Luxemburger C, Thwai KL, White NJ, Webster HK, Kyle DE, Maelankirri L, Chongsuphajaisiddhi T, Nosten F, 1996. The epidemiology of malaria in a Karen population on the western border of Thailand. *Trans R Soc Trop Med Hyg 90:* 105–111.
10. Taylor Robinson AW, Smith EC, 1999. A dichotomous role for nitric oxide in protection against blood stage malaria infection. *Immunol Lett 67:* 1–9.
11. Jacobs P, Radzioch D, Stevenson MM, 1996. In vivo regulation of nitric oxide production by tumor necrosis factor alpha and gamma interferon, but not by interleukin-4, during blood stage malaria in mice. *Infect Immun 64:* 44–49.
12. Angele MK, Smail N, Knoferl MW, Ayala A, Cioffi WG, Chaudry IH, 1999. L-arginine restores splenocyte functions after trauma and hemorrage potentially by improving blood flow. *Am J Physiol 276:* 45–51.
13. Silamut K, White NJ, 1993. Relation of the stage of parasite development in the peripheral blood to prognosis in severe falciparum malaria. *Trans R Soc Trop Med Hyg 87:* 436–443.
14. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR, 1982. Analysis of nitrate, nitrite, [15N]nitrate in biological fluids. *Ann Biochem 126:* 131–138.
15. Gupta S, Hill AV, Kwiatkowski D, Greenwood AM, Greenwood BM, Day KP, 1994. Parasite virulence and disease patterns in P*lasmodium falciparum* malaria. *Proc Natl Acad Sci USA 91:* 3715–3719.
16. David PH, Hommel M, Miller LH, Udeinya IJ, Oligino LD, 1983. Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. *Proc Natl Acad Sci USA 80:* 5075–5079.
17. Hermsen CC, Mommers E, van de Wiel T, Sauerwein RW, Eling WM, 1998. Convulsions due to increased permeability of the blood-brain barrier in experimental cerebral malaria can be prevented by splenectomy or anti T-cell treatment. *J Infect Dis 178:* 1225–1227.

18. Quinn TC, Wyler DJ, 1980. Resolution of acute malaria (*Plasmodium berghei* in the rat): reversibility and spleen dependence. *Am J Trop Med Hyg 29:* 1–4.
19. Wyler DJ, 1983. Splenic functions in malaria. *Lymphology 16:* 121–127.
20. Weiss L, 1989. Mechanisms of splenic control of murine malaria: cellular reactions of the spleen in lethal (strain 17XL) *Plasmodium yoelii* malaria in BALB/c mice, and the consequences of pre-infective splenectomy. *Am J Trop Med Hyg 41:* 144–160.
21. Nacher M, Gay F, Singhasivanon P, Krudsood S, Treeprasertsuk S, Mazier D, Vouldoukis I, Looareesuwan S, 2000. *Ascaris lumbricoides* infection is associated with protection from cerebral malaria. *Parasite Immunol 22:* 107–114.