CYTOKINES ASSOCIATED WITH PATHOLOGY IN THE BRAIN TISSUE OF FATAL MALARIA

Yaowapa Maneerat[1], Emsri Pongponratn[1], Parnpen Viriyavejakul[1], Benjanee Punpoowong[1], Sornchai Looareesuwan[2], and Rachanee Udomsangpetch[3]

[1]Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [2]The Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [3]Department of Pathobiology, Faculty of Science, and The Graduate Studies, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand

Abstract. Cytoadherence of *Plasmodium falciparum*-infected erythrocytes to the brain microvascular endothelial cells is believed to be an important cause of circulatory blockage in cerebral malaria. Cytokines released during acute infection may activate brain endothelial cells leading to increased binding of infected erythrocytes in the brain and reduced cerebral blood flow. This effect may be direct and more potent with the tissue-localized cytokines in the brain. In order to establish this relationship, brain tissues of cerebral and noncerebral malaria were compared. The most prominent histopathologic changes in the brain included edema, neuronal degeneration, ring hemorrhage, and percentage of parasitized erythrocytes sequestration were observed in cerebral malaria. Immunohistochemical staining of the brain sections demonstrated that tissue-localized TNF-α, IFN-γ, IL-1β, and IL-10 were associated with the histopathology. However, IL-4 was the only cytokine presented at moderate level in the brain tissue of noncerebral malaria which histopathology was the least. No tissue-localized cytokine was observed in the brain of *P. vivax* infection or of the car accident control cases.

INTRODUCTION

Malaria remains one of the serious public health problems in tropical countries. The important cause of severe malaria in human is *Plasmodium falciparum* infection which leads to cerebral complications and death (Mendis and Carter, 1995). Sequestration of the erythrocytes containing mature forms of *P. falciparum* in capillaries may play a pathogenic role (Pongponratn *et al*, 1991). Two properties of *P. falciparum*-infected erythrocytes involved in the vascular obstruction are cytoadherence (Barnwell *et al*, 1989) and rosetting (Udomsangpetch *et al*, 1989). Histopathology of the brain and other organs in human cerebral malaria is well described. However, there is no appropriate animal model for demonstrating the mechanism of histopathogenesis of cerebral malaria. Immunologically, *P. falciparum* antigens are shown to stimulate cytokine production *in vitro* by different cell types including monocytes (Kwiatkowski *et al*, 1990) and endothelial cells (Xiao *et al*, 1995). Elevation of plasma cytokines, *ie* TNFα, IFN-γ, IL-1 (Kwiatkowski *et al*, 1990), and IL-6 (Kern *et al*, 1989) has been associated with the severity of falciparum malaria. *In vitro* studies also show that cytokine-activated endothelial cells increase expression of adhesion proteins such as intercellular adhesion molecule 1 (ICAM-1) (Pober *et al*, 1987) which is one of the receptors for parasite adherence (Berendt *et al*, 1989). A recent report has shown TNFα in macrophages but not in parenchyma of brain tissues from a cerebral malaria patient with HIV coinfection (Porta *et al*, 1993). However, the role of cytokines in histopathogenesis of cerebral malaria is not understood. Our recent study has shown cytokines localized in the brain tissue of cerebral malaria in contrast to that observed in the normal brain tissue (Udomsangpetch *et al*, 1997). We, therefore, investigated further i) whether cytokines in the brain tissues are different in cerebral and noncerebral malaria, and ii) whether the levels and types of tissue-localized cytokines are associated with brain histopathology in cerebral malaria.

Correspondence: Dr Yaowapa Maneerat, Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Rajvithi Road, Bangkok 10400, Thailand. Fax: 662-2468340; E-mail: tmymn@mahidol.ac.th

Reprint requests: Dr Rachanee Udomsangpetch, Department of Pathobiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand. E-mail: scrud@mahidol.ac.th

MATERIALS AND METHODS

Patient history

Four severe patients with *P. falciparum* infec-

tion who were admitted and died at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University during 1985 to 1990 were studied. These severely ill patients were defined as cerebral (cases No. 1 and 2) and non-cerebral malaria (cases No. 3 and 4) according to the clinical measurements and coma scale (Warrell *et al*, 1988). Patients with cerebral malaria were defined as unrousable coma with positive asexual forms of falciparum malaria in the peripheral blood and other causes of coma were excluded (Warrell *et al*, 1982, 1988). Cases No.1 and 2 had coma scale = 2 (conciousness was unrousable and non localizing motor response); cases No. 3 and 4 had coma scale = 0 (rousable to fully conciousness). The clinical and laboratory findings of the four patients are summarized in Table 1.

Case No. 1: A 54 years old male had a history of one malaria infection a year ago before this infection. Six days prior to admission the patient developed fever with chill and headache. On admission day the patient was in comatose state due to *P. falciparum* infection and respiratory failure. Giemsa-stained blood films confirmed *P. falciparum* malaria with 78 ring form and 12 gametocytes/1,000 RBC. The patient was treated with quinine and supportive drugs to improve metabolic acidosis. Finally the patient developed spasm and cardiac arrest and died 14 hours after admission.

Case No. 2: A 18 years old male had lived in the malaria endemic area in the central part of Thailand for 6 months. The patient had fever with chill, jaundice, nausea, vomiting, liver enlargement, and was misdiagnosed as acute hepatitis at a rural hospital. Later the patient developed tonic convulsions, went into coma and was transferred to the Hospital for Tropical Diseases. Blood smear confirmed *P. falciparum* malaria with 10 ring forms, 1 schizont and 1 gametocyte/1,000 RBC. The patient was treated with quinine but did not improve and died within 6 hours of admission with coma and respiratory failure.

Case No. 3: A 65 years old male had worked in an endemic area in the south of Thailand for 2 years. The patient had had a history of malaria infection and used to be treated at the local malaria clinic. On admission at the Hospital for Tropical Diseases the patient had low fever with chill for 8 days, was in dehydrated status with signs of pulmonary edema and hypotension. Blood examination showed 3 ring forms of *P. falciparum*/200 WBC. The patient was given fansidar, quinine and antipyretic drugs. Physical examination revealed that the patient also had tuberculosis. He did not recover but developed dyspnea and died from respiratory failure on the fifth day after admission.

Table 1
Clinical and laboratory features of patients on admission.

Clinical and laboratory features	Case 1	Case 2	Case 3	Case 4
Coma scale*	2	2	0	0
Temperature (°C)	39.0	38.2	37.5	39.0
Blood pressure (mm Hg)	110/60	150/50	100/70	110/60
Respiratory rate/minute	28	48	NA	22
Pulse/minute	110	124	84	100
WBC x 10^9/liter	32	28.2	9.3	9.5
Hematocrit (%)	33	22	35	29
Parasite density/μl	314,120	29,900	9,140	41,550
Schizont/200 WBC	0	1	0	1
Gametocyte/200 WBC	0	1	0	2
Blood glucose (mg%)	254	98	87	NA
Blood urea nitrogen (mg%)	96	NA	18.8	9.3
Creatinine (mg%)	5.0	NA	1.47	1.2
Bilirubin				
Total (mg%)	5.94	NA	0.3	1.83
Direct (mg%)	2.55	NA	0.12	0.31

* Cerebral malaria coma scale established by Warrell *et al*, 1988.
NA= not available

Case No. 4: A 21 years old female had worked in an endemic area near the western border of Thailand and had taken antimalarial drugs for malaria prophylaxis regularly for 1 year. On a visit to the local clinic, the patient had fever, chill, malaise, nausea, vomiting, and was misdiagnosed and treated as a simple cold. The patient did not recover and was transferred to the Hospital for Tropical Diseases. Physical examination revealed that the patient was 7 $^{1}/_{2}$ months pregnant, had normal heart and lung, good conscious and mild icteric sclera. Blood film examinations showed 24 ring forms/1000 RBC, 1 schizont and 2 gametocytes/200 WBC. The patient was treated with quinine and supportive treatment to maintain electrolyte balance. During admission the patient had prolonged fever, developed pneumonia and was treated with 1 g of ampicillin and antipyretic drugs. Metabolic acidosis developed and the patient died of acute respiratory failure and cardiac arrest on the fourth day after admission.

Case No.5: (*P. vivax* -infected patient): The splenectomized patient was a 16 year old male who presented with fever and body malaise for 2 days. On admission, he had fever with dyspnea, mild jaundice and mild dehydration. Malaria screening was positive for *P. vivax* (2 AV/1,000 RBC). One day after admission, his consciousness deteriorated and he developed metaboloic acidosis, diarrhea, deep jaundice and acute renal failure. He died 2 days after admission because of metabolic acidosis, septicemia and acute renal failure.

Brain tissue preparations

Following consent of the patient's relatives, autopsy examinations of all cases were performed during 1985-1990 at the Department of Tropical Pathology, Faculty of Tropical Medicine, Bangkok, Thailand. Small pieces of brain tissues (cerebrum) were fixed in 10% buffered formalin for at least 24 hours and embedded in paraffin. Control brain tissues previously collected from two autopsied car accident cases were included in the study. Only 1 necropsied tissue was obtained from the *P. vivax*-infected case. As for positive control of cytokine production in the tissue, sublingual tonsil from two cases of tonsillitis were used.

Table 2
Comparison of pathological findings in the brain tissue of cerebral and noncerebral malaria.

Group of severity	Parasitized red cell sequestration (% of microvessels)	Degree of edema	Ring hemorrhage	Mononuclear cell infiltration
Cerebral				
No.1	100	none	none	none
No.2	100	moderate	numerous	rare*
Noncerebral				
No.3	0	mild	none	none
No.4	0	none	none	none

*Only few mononuclear cells infiltrated the brain parenchyma whereas numerous mononuclear cells packed in some vessels.

Table 3
Cytokines in the brain tissues of cerebral and noncerebral malaria.

Group of severity	TNF-α	IFN-γ	IL-1β	IL-4	IL-10
Cerebral					
No.1	++	++	++	+	++
No.2	++	++	++	+	++
Noncerebral					
No.3	+	+	+	++	+
No.4	-	+	+	++	+

Note: Cytokines were determined by indirect immunofluorescence assay as described in Materials and Methods. Intensity of the staining was scored as - = no staining, + = weak, ++ = moderate, and +++ = extensive staining.

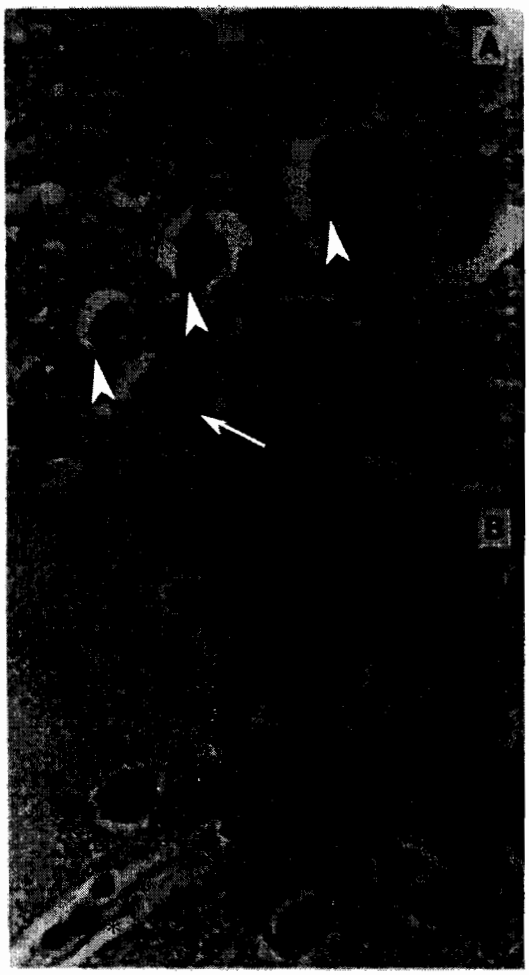

Fig 1–Brain sections show differences in histopathologic change between cerebral malaria case No. 2 (A) and noncerebral malaria case No. 3 (B). Edema of the brain tissue is indicated by the space (arrows) between nerve fibers in stroma, and widening of the Virchow-Robin space (asterisk), degenerative neurons (arrow heads) are also shown. (Hematoxylin and eosin staining, x400).

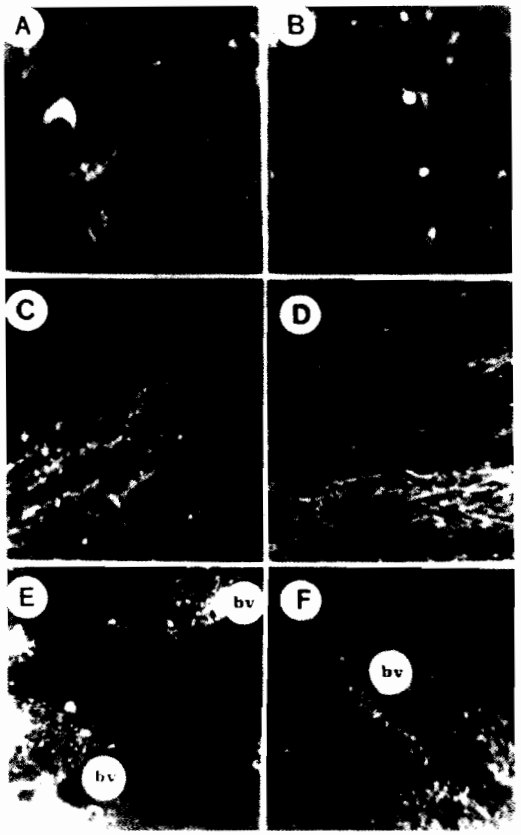

Fig 2–Immunofluorescence staining of cytokines in the brain sections from cerebral malaria. Cerebral malaria case No. 2 shows (A) TNF-α in neuron (x200) compared with (B) negatively stained neuron (x400) in a noncerebral malaria case No. 3; (C, x400) and (D, x200) show TNF-α and IL-10, respectively, in the nerve fiber of case No. 2, (E) focal staining of IFN-γ in the white matter of case No. 2 (x200), (F) IL-4 in the white matter of noncerebral malaria case No. 3 (x200); > = neuron, bv = blood vessel.

Histopathology

The paraffin-embedded tissues were sectioned at 7 mm thick, and routinely processed for staining with standard Mayer's hematoxylin and eosin. Histopathology of all tissue sections was carefully examined by light microscope. The brain tissues of cerebral and non-cerebral malaria cases were then compared.

Parasitized erythrocyte (PRBC) sequestration in the brain was quantified by examining 100 cerebral microvessels in each case and the percentages of sequestration were then compared.

Monoclonal antibodies

Primary antibodies: Murine monoclonal antibodies (mAb) to cytokines including TNF-α, IFN-γ, IL-1β, IL-4, IL-10 were obtained from Genzyme Diagnostics, MA, USA.

Secondary antibody: $F(ab)_2$ fraction of goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) was obtained from Sigma, MO, USA.

Indirect immunofluorescence assay

Brain tissue sections of all the cases, and sections of tonsils were deparaffinized at 50°C for at least 4 hours, rinsed in xylene twice for 5 minutes each, and gradually rehydrated twice in absolute ethyl alcohol, 90% and 70% ethyl alcohol for 5 minutes each, then rinsed gently in running tap water for 5 minutes, and rinsed briefly with 0.1 M phosphate-buffered saline (PBS) before used in immunostaining. The brain sections were incubated with an optimal concentration (40 μg/ml) of mAb to each cytokine in a humidified chamber for 30 minutes at room temperature. The sections were washed 3 times, 5 minutes each, in 0.1 M PBS, then incubated with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG at 1:100 dilutions for 30 minutes, washed as above and mounted with fluorescent mounting medium (DAKO Corporation, CA, USA). The brain sections were then examined under a fluorescence microscope. Intensity of the staining was scored as follows: - = very little or no staining, + = weak, ++ = moderate, and +++ = extensive staining.

RESULTS

Histopathology

Histopathologic changes of the brain tissues between cerebral, noncerebral malaria cases, and negative controls are compared in Fig I and Table 2.

Cerebral malaria: Cerebral edema, determined by the gross presence of uncal hernia in post-mortem brain and histological widening of the Virchow-Robin space (Fig 1A), was observed only in the brain of case No. 2. Although all cerebral capillaries in the brain sections of the two cerebral malaria cases were congested with PRBC (100% sequestration), more severe histopathologic changes were observed in case No. 2 (Table 2). These histopathologic changes included some degenerative neurons (Fig 1A), ring or perivascular hemorrhages were observed in the white matter.

Noncerebral malaria: Brain sections of the two noncerebral malaria cases showed no PRBC sequestration. No hemorrhage or cerebral edema was seen. Only congestion of uninfected erythrocytes in the vessels was observed in the brain and meninges of both cases. Nerve cells, glial cells and stroma were essentially normal (Fig 1B).

Non *P. falciparum* infection: Brain sections of the two car accident cases showed minor pathologic changes and no inflammatory cell infiltration except some congestion in the blood vessels. Nerve cells and supporting cells showed good appearance. Brain sections of the *P. vivax* infected case also showed little pathologic changes including congestion in some vessels but no petichial haemorrhage, edema or neuronal degeneration.

Determination of tissue cytokines

Indirect immunofluorescence assay was used to determine the presence of cytokines in the brain tissues of cerebral and noncerebral malaria in comparison to that in the control tissues. The fluorescence intensity of the staining of these cytokines was compared semiquantitatively (Table 3) in order to elucidate an association between the two malaria groups.

Cerebral malaria: Cytokines found in the brain tissues were TNF-α, IFN-γ, IL-1β, and IL-10. The intensity of fluorescence staining was moderate. Distribution of these cytokines was typical. TNF-α was found in the nerve cells and filamentous tissues (Fig 2A) compared to the negative staining area of the same tissues (Fig 2B). IL-1β (Fig 2C) and IL-10 (Fig 2D) were found in the bundles of filaments like nerve fibers. IFN-γ was found in the white matter area adjacent to either the blood vessels or the nerve fibers (Fig 2E). Weak staining of IL-4 was also found in filamentous tissue (not shown).

Noncerebral malaria: In contrast to those found in the cerebral malaria, moderate fluorescence staining intensity of IL-4 (Fig 2F) was observed in the area adjacent to blood vessels. A weak staining of TNF-α, IFN-γ, IL-1β, and IL-10 was occasionally seen in the nerve fibers and around some blood vessels (not shown).

Negative controls: There was only weak fluorescence staining of IL-4 and IL-10 in a few neurons of the car accident brain sections. There were no cytokines found in the brain sections of the *P. vivax* case (not shown).

Positive controls: The positive reactivity of indirect immunofluorescence assay was confirmed by using lymphoid tissue sections from 2 cases of tonsillitis. A moderate fluorescence staining of IL-1β and IFN-γ were found in some mononuclear cells in the lymphoid follicles.

DISCUSSION

Histopathology of the brain tissues of cerebral

malaria compared to noncerebral malaria confirmed that the percentage of parasite sequestration in the brain microvessels was a striking difference between the two malaria groups (Pongponrarn *et al*, 1991). Although cerebral malaria case No. 1 had a greater percentage of parasitemia at admission, the brain tissues of case No. 2 had more changes. This confirmed a previous report that parasite sequestration alone does not account for the cerebral complication in *P. falciparum* infection (Turner, 1997). However, neuronal degeneration observed in these *P. falciparum*-infected cases could be the result of the disease rather than post mortem changes as judged by comparison with the brain tissues from the two car accident cases and the *P. vivax*-infected case. Although the two cases of noncerebral malaria were fully conscious upon admission, the disease complication developed rapidly and these patients died of secondary infection. The good histologic appearance in the brain sections of these patients corresponded well with their consciousness and low severity of *P. falciparum* infection.

By indirect immunofluorescence staining, we demonstrated the expression of TNF-α, IFN-γ, IL-1β, and IL-10 in the brain tissues of cerebral malaria. These observations were consistent with our previous study using brain tissues from another two cerebral malaria cases (Udomsangpetch *et al*, 1997). Additionally, the present study showed a different pattern of tissue cytokine (IL-4 expression) in noncerebral malaria compared with that of cerebral malaria. Brain tissue pathogenesis in cerebral malaria was associated with intensity of cytokine staining, unlike the minor changes observed in noncerebral malaria cases. Tissue-associated IL-1β in our study was consistent with the role of TNF-α in stimulation of IL-1β production (Dinarello and Wolff, 1993). Our observation was supported by a recent study in mice infected by human babesia, *Babesia microti* or WA1, which demonstrates pathogenic effects of cytokines. The fatal WA1-infected mice increase production of TNF-α and IFN-γ in the brain throughout the infection. The surviving *B. microti*-infected mice, on the other hand, increase production of TNF-α, IL-10 and IL-4 early in the infection, and decrease the levels during late infection (Hemmer *et al*, 1996). The differences of cytokines found in the brain tissues between cerebral and noncerebral malaria were not only the level but also the type of cytokines (TNF-α, IFN-γ, IL-1β, IL-10) related to histopathogenesis in *P. falciparum*-infected humans.

In the present study, it was observed that neurons and glial cells produced these tissue cytokines for the clear fluorescence staining was observed in these cells and the nerve fibers. The ability of neurons to produce TNF-α (Liu *et al*, 1994) and of glial cells to produce IL-1β (Yabuchi *et al*, 1994) have been shown previously.

Although much about malaria pathogenesis remains to be unraveled, the results of this study add more information to the understanding on the involvement of cytokines in histopathogenesis of cerebral malaria in humans.

ACKNOWLEDGEMENTS

We are grateful to the staff at the Hospital for Tropical Diseases, Mahidol University for assistance with the autopsies, to Dr M Riganti for discussion on malaria pathology, to Dr G Turner for technical advice and discussion, and to Professor NJ White for his constructive comments. Tissue preparations by Mr K Bophan and Mr V Khachansaksumeth are most appreciated. This work received financial support from the Thailand Research Fund (TRF) and partial support from the NIH grant U01-A135827-01. YM is a TRF-Postdoctoral Fellow.

REFERENCES

Barnwell J, Asch A, Nachman R, *et al.* Human 88 kDal membrane glycoprotein (CD36) functions *in vitro* as a receptor for a cytoadherence ligand on *Plasmodium falciparum*-infected erythrocytes. *J Clin Invest* 1989; 84: 1054-61.

Berendt AR, Simmons DL, Tansey J, *et al.* Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. *Nature* 1989; 34: 57-9.

Dinarello CA, Wolff SM. The role of interleukin-1 in disease. *N Engl J Med* 1993; 328: 106-13.

Hemmer RM, Ferrick DA, Conrad PA. Roles of cytokines in the pathogenesis of human babesia isolates in mice [Abstract]. The 45th Annual Meeting of The American Society of Tropical Medicine and Hygiene, Maryland, December 1-5, 1996: 235.

Kern P, Hemmer CJ, Damme JV, *et al.* Elevated of tumor necrosis factor alpha and interleukin-6 serum levels as a markers for complicated *Plasmodium falciparum* malaria. *Am J Med* 1989; 87: 139-43.

Kwiatkowski D, Hill AVS, Sambou I, *et al.* TNF concentration in fatal cerebral, non fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet* 1990; 336: 1201-4.

Liu T, Clark RK, McDonnell PC, *et al.* Tumor necrosis factor-alpha expression in ischemic neurons. *Stroke* 1994; 25: 1481-8.

Mendis KN, Carter R. Clinical disease and pathogenesis in malaria. *Parasitol Today* 1995; 11: 2-16.

Pober JS, Lapierre LA, Stolpen AH, *et al.* Activation of cultured endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. *J Immunol* 1987; 138: 3319-24.

Pongponratn E, Riganti M, Punpoowong B, *et al.* Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: A pathological study. *Am J Trop Med Hyg* 1991; 144: 168-75.

Porta J, Carota A, Pizzolato G, *et al.* Immunopathological changes in human cerebral malaria. *Clin Neuropathol* 1993; 12: 142-6.

Turner GDH. Cerebral malaria. *Brain Pathol* 1997; 7: 569-82.

Udomsangpetch R, Chivapat S, Viriyavejakul P, *et al.* Involvement of cytokines in the histopathology of cerebral malaria. *Am J Trop Med Hyg* 1997; 57: 501-6.

Udomsangpetch R, Wahlin B, Carlson J. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocytes rosettes. *J Exp Med* 1989; 169: 1835-40.

Warrell DA, Looareesuwan S, Warrell M. Dexamethasone proves deleterious in cerebral malaria [letter]. *Br Med J* 1982; 285: 1652.

Warrell DA, White NJ, Veall N, *et al.* Cerebral anaerobic glycolysis and reduced cerebral oxygen transport in human cerebral malaria. *Lancet* 1988; 2: 534-8.

Xiao L, Yang C, Saekhou AM, *et al.* Cytokine production by endothelial cell after stimulation with *Plasmodium falciparum* blood stage antigen [Abstract]. The 44th Annual Meeting of the American Society of Tropical Medicine and Hygiene, San Antonio, Texas, 1995: 93.

Yabuchi K, Minami M, Katsumata S, *et al.* An *in situ* hybridization study on interleukin-1 beta mRNA induced by transient forebrain ischemia in the rat brain. *Brain Res Mol Brain Res* 1994; 26: 135-42.