PROGNOSTIC SIGNIFICANCE OF SKIN AND SUBCUTANEOUS FAT SEQUESTRATION OF PARASITES IN SEVERE FALCIPARUM MALARIA

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Abstract. Intradermal blood smear, histopathologic and immunohistologic studies were performed in severe malaria (n=10) and uncomplicated malaria (n=10) patients during positive parasitemia and within 6 hours after negative parasitemia by finger prick smears. Intradermal blood smears showed asexual forms and intraleukocytic pigments when finger prick blood smears showed negative results; however intradermal blood smear did not indicate disease severity within 6 hours after negative parasitemia by finger prick. Histopathologic findings showed 15 fold higher parasitized red blood cells sequestered in vessels of subcutaneous fatty tissue in severe malaria than in uncomplicated malaria ($p<0.001$) and may indicate disease severity. A panel of epithelial antibodies against cytokines applied to skin biopsies clearly detected a higher titer against tumor necrosis factor-alpha (TNFα) and interleukin-10 (IL-10) in dermal vessels and collagen respectively, in severe malaria compared with uncomplicated malaria. Results of the study suggest that histopathology and immunohistology of skin and subcutaneous fatty tissue may indicate prognostic severity of malaria and may be associated with focal accumulation of cytokines.

INTRODUCTION

Infection with *Plasmodium falciparum* remains a major cause of death in the tropics, with an annual global mortality of 1-2 million people and a mortality rate in severe malaria of 15-30% (WHO 1990, 1993). The sequestration of red blood cells (RBC) containing the mature forms of the parasite in the microcirculation of the vital organs is considered to be an essential pathogenic element of severe infection, but precisely how this causes death is not known. Sequestration interferes with microcirculatory flow and tissue metabolism and may focus the release of host- or parasite-derived toxins to the vital organs (White and Ho, 1992). The cytoadherent parasitized red blood cells (PRBC) impede the passage of the uninfected RBC, which are forced to deform more than usual in their transit through the microcirculation.

It has been suggested that intradermal smear taken from the volar aspect of the forearm, a technique described originally by van der Berghe and Chardome (1951), may be positive for malaria parasites after the peripheral blood has become negative. Nakano *et al* (1996) reported that rhesus monkeys infected with *Plasmodium coatneyi* showed parasitized erythrocyte sequestration in microvessels of the brain very similar to that seen in human patients with cerebral malaria, and confirmed that *P. coatneyi*-infected monkeys could be used as an animal model for the study of human cerebral malaria. This study showed that it was consistent with the finding of parasitized erythrocyte sequestration in subcutaneous tissues in a comatose monkey and suggested biopsy specimens of subcutaneous tissue might be useful as indicators of parasitized erythrocyte sequestration in the brain of cerebral malaria patients. The study also suggested that biopsy of subcutaneous tissues might be useful for the determination of the severity (Mokken *et al*, 1992) and prognosis of human cerebral malaria.

Udomsangpetch *et al* (1997) studied 2 fatal patients with cerebral malaria and found TNFα, interferon-gamma (IFNγ), IL-1β and IL-10 in brain and liver but not in skin. Thus those cytokines may be found in severe malaria. However, more

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live severe patients should be studied for a better understanding of cytokine involvement in severe malaria.

Although several prognostic factors have been identified in severe malaria, including depth of coma, hyperparasitemia, the predominance of late stages of parasite development or a high proportion of neutrophils containing malaria pigments, hypoglycemia, elevated plasma levels of tumor necrosis factor, elevated lactate levels in the blood and cerebrospinal fluid, and the severity of acidosis (WHO, 1990; White and Ho, 1992), there has been no study of prognostic significance of skin and subcutaneous erythrocyte sequestration and tissue cytokines in live severe malaria patients by skin biopsy.

The objectives of the study were to demonstrate whether intradermal blood smear and skin and subcutaneous fatty tissue (SFT) sequestration of *P. falciparum* erythrocytes by skin biopsy can be used for diagnosis of severe falciparum malaria after disappearance of parasitemia determined by finger prick, and to determine cytokine involvement in skin histopathology.

MATERIALS AND METHODS

Patients

Ten patients with severe falciparum malaria according to WHO criteria (1990) and ten uncomplicated falciparum malaria patients admitted to the Hospital for Tropical Diseases, Bangkok, Thailand were studied between July and November 1999. No patient had a previous history of skin disease, allergic disease or taking antihistamine or immunosuppressive drug before entering the study. Clinical, biochemical, and hematologic details of the patients are given in Table 1. Patients remained in the ward until either death or complete clearance of parasites. Treatment with antimalarial drugs before admission to the study was not an exclusion criterion.

Methods

Skin biopsy, skin squeezing for intradermal blood smear and skin biopsy for histopathology and immunopathology were done on admission when the patients had positive parasitemia by finger prick. Capillary blood samples were obtained by pricking the finger pulp with a 25-gauze needle every 6 hours until negative parasitemia are detected. Positive and negative parasitemia by finger prick in this study were defined as the positive and negative findings of asexual forms of *P. falciparum* by finger prick smears, respectively. Within 6 hours after negative parasitemia by finger prick, skin squeezing for intradermal blood smear and skin biopsy for histopathology and immunopathology were studied again. Informed consent to skin biopsy was obtained from the patients or their accompanying relatives.

Collection and fixation of specimens

Finger prick smear : The finger which was selected for pricking was thoroughly cleaned with 70% alcohol. The finger was pricked and gently squeezed and the upper and lower side of the finger selected between the thumb and index finger just below the pricking point by continuous gentle pressure. The subsequent drops of blood were dropped on the slide and the smear of blood were made both in thin and thick films. Giemsa was used for staining thick and thin smears.

Skin biopsy : For light microscopy, skin was biopsied at the area of the arm above the elbow with a standard 4-mm circular punch. Care was taken not to squeeze the skin beforehand. Local anesthetic, 1% xylocain, was infiltrated subcutaneously. Skin biopsy was done beyond the needle puncture site (for xylocain infiltration). The specimens were fixed in 10% buffered neutral and formalin, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin, Giemsa and Thomas. The tissue sections were examined using a Nikon Optiphot-2 photomicroscope. Blood vessels were identified, intravascular erythrocytes were counted, and the proportion containing recognizable asexual parasites was scored. At least 20 blood vessels (or all vessels on small or sparsely vascularized specimens) were identified and scored for a variety of characteristics.

Interdermal smear : The site of puncture was at the posterior surface of the elbow joint about 5-7 cm from the tip of olecranon. It was thoroughly cleaned, first with cotton soaked with 70% alcohol and then with dry cotton to remove all traces of alcohol. The skin was fixed between the thumb and the index finger and a puncture was made horizontally to the skin at 5 points in an area of 4 mm in diameter and 1 mm in depth. The blood was gently squeezed out of each point and then the smear was made (Li *et al*, 1989). Giemsa stain was applied for staining thick and thin smears.

The biopsy site taken at a different site from the intradermal smear was studied. Light microscopy was used to determine the asexual forms of malaria parasites per 1,000 erythrocytes for the thin blood smear and per 200 leukocytes for the thick blood smear. If no parasite was seen by thick smear examination, continuing examination to all slide fields was done, whereas if no parasite was seen by thin smear examination, examination was stopped. Slides were read by one expert technician who was blinded as to the identity and clinical details of the patients.

Preparation of tissues for immunofluorescence staining : Fresh frozen sections of skin biopsies were prepared and fixed with cold acetone 15 minutes, dried and stained with rabbit antisera specific to IFNγ, TNFα, IL-1β, IL-2, IL-6, IL-10, granulocyte-macrophage colony-stimulationg factor (GM-CSF)) and factor 8. The sections were incubated with $F(ab')_2$ fragment for fluorescein isothocyanate (FICT)-conjugated anti-rabbit immunoglobulin. The stained sections were examined under a UV-light microscope. Skin sections incubated with fluorescein conjugate alone were used as controls for staining specificity.

Statistical analysis

For comparison of groups, the Mann-Whitney *U* test was applied, and the a level was set at 0.05. Proportions were compared by use of the chi-squared test with Yates correction or Fischer's exact test as appropriate. All statistical analyses were performed by use of the statistical computing package SPSS for Windows (SPSS, Chicago) and Epi Info-6.04b (CDC, Atlanta).

RESULTS

Patient characteristics

Ten severe malaria and ten uncomplicated malaria patients were enrolled. All patients had positive parasitemia by finger prick thick and thin smear. Skin biopsy and intradermal blood smear (squeezing) were carried out on the day of admission (as study entry with positive parasitemia) and again within 6 hours after negative parasitemia by finger prick smear. After entering the study, finger prick was done every six hours until negative parasitemia. Then the second skin biopsies were done in all patients. Only one severe patient had a third skin biopsy 14 days after the second biopsy and at that time the patient had negative parasitemia by finger prick. All patients were male without previous underlying diseases. There were no patient deaths. All severe patients were treated with artemisinin derivatives. Nine uncomplicated patients were also treated with artemisinin derivatives followed by mefloquine; and the tenth uncomplicated patient was treated with quinine and tetracycline.

Clinical and laboratory parameters

Clinical and laboratory baseline data are shown in Table 1. The ages of severe and uncomplicated patients were similar. There were significant differences in fever clearance time (FCT), blood urea nitrogen, creatinine, total bilirubin, aspartate aminotransferase, alanine aminotransferase and albumin between severe and uncomplicated patients ($p \leq 0.001$, 0.03, 0.02, 0.002, < 0.001, < 0.001 and 0.007, respectively). Cerebral malaria, acute renal failure, jaundice, hyperparasitemia and anemia were complications found only in severe patients. Nine patients in the severe group had multiple or more-than-one complications. Schizontemia was found in 7 patients (70%) in the severe group whereas no schizontemia was observed in the uncomplicated group.

In severe malaria, FCT was similar to parasite clearance time (PCT) whereas in uncomplicated malaria FCT was significantly shorter than PCT ($p < 0.001$). FCT was significantly longer in severe malaria than in uncomplicated malaria ($p = 0.04$). PCT was similar in severe and uncomplicated groups. Although the geometric means of parasitemia and leukocyte count in severe malaria were higher than in uncomplicated malaria, they showed no significant difference.

Histopathology (Table 2; Figs 1,2)

Comparing severe malaria with uncomplicated malaria in the first biopsy, there were significant differences in % PRBC in vessels of subcutaneous fatty tissue (VSFT), tightness of packing of RBC in VSFT, % VSFT with 2+ packing and % vessels containing PRBC in SFT ($p = 0.001$, 0.03, 0.03 and 0.02, respectively). On admission, PRBC were more frequently found in VSFT in severe malaria than in uncomplicated malaria.

Comparing the first biopsy with the second biopsy in servere malaria, there were significant differences in % PRBC in VSFT, % VSFT with 2+ packing and % vessels containing PRBC in SFT ($p = 0.001$, 0.04, and 0.02, respectively). PRBC

Table 1
Patient demographic data.

	Severe malaria (n = 10)	Uncomplicated malaria (n = 10)	p-value
Age (years)	26.90 ± 11.70	24.9 ± 10.47	NS
FCT (hours)	182.60 ± 87.09	21.1 ± 14.93	<0.001
PCT (hours)	52.20 ± 13.37	48.8 ± 12.04	NS
Parasite count [geometric mean (range) (/μl)]	56,234 (82-595,200)	38,904 (4,840-97,760)	NS
PCV (%)	30.40 ± 9.55	37.9 ± 7.26	NS
WBC (/μl)	10,000.00 ± 4,836.44	6,880 ± 2,640.2	NS
BUN (mg/dl)	49.35 ± 39.62	18.45 ± 10.12	0.03
Cr (mg/dl)	1.74 ± 1.37	0.82 ± 0.42	0.02
TB (mg/dl)	5.09 ± 3.47	1.45 ± 0.75	0.002
AST (IU/l)	216.50 ± 206.44	26.2 ± 8.28	<0.001
ALT (IU/l)	163.60 ± 195.59	30.6 ± 17.69	<0.001
Albumin (g/dl)	3.01 ± 0.65	3.9 ± 0.58	0.007
Cerebral malaria	5 (50%)	0	
Renal failure	3 (30%)	0	
Jaundice	9 (90%)	0	
Hyperparasitemia	6 (60%)	0	
Schizontemia	7 (70%)	0	
Severe anemia	3 (30%)	0	

FCT=fever clearance time; PCT=parasite clearance time; PCV=packed cell volume; WBC=white blood cells; BUN=blood urea nitrogen; Cr=creatinine; TB=total bilirubin; AST=aspartate aminotransferase; ALT=alanine aminotransferase

were more frequently found in VSFT in severe malaria on admission with positive parasitemia from finger prick than in severe malaria after treatment with negative parasitemia. Comparing severe malaria with uncomplicated malaria in the second biopsies, there was a significant difference in the % of vessels containing PRBC in SFT (p=0.04). PRBC were more frequently seen in severe malaria than in uncomplicated patients. Comparing findings of the first biopsy with the second biopsy in uncomplicated malaria, they showed no significant difference. Within 6 hours after blood smear from finger prick showing negative parasitemia, PRBC were frequently still found in vessels of the skin and subcutaneous tissues, particularly VSFT.

Comparing % PRBC sequestered in VSFT (67.8±25.66%) with % PRBC in peripheral blood by finger prick (6.04±6.85%) of severe malaria in the first biopsy, they showed a significant difference ($p < 0.001$). Comparing % PRBC sequestered in VSFT (4.5±2.84%) with % PRBC in peripheral blood by finger prick (1.13±0.73%) in the first biopsy of uncomplicated malaria, they showed a significant difference ($p < 0.001$). There were 11 and 4 fold more PRBC sequestered in VSFT than in peripheral blood in severe and uncomplicated patients respectively. In the first biopsy, % PRBC sequestered in VSFT were 15 fold higher in severe malaria than in uncomplicated malaria (67.8±25.66% *vs* 4.5±2.84%; $p < 0.001$).

The third skin biopsy was done in one severe malaria patient 14 days after the second biopsy. At that time the patient was asymptomatic. Thick smear by finger prick showed 156 gametocytes/μl. Skin biopsy showed diapedesis in the deep dermis and SFT, PRBC, 1% PRBC in VSFT, 2+ score of tightness of packing of RBC in VSFT, 2% score of VSFT with 2+ packing, 1% of vessels containing PRBC in SFT, presences of leukocytes, platelets, gametocytes, endothelial cell ingesting of malarial pigments. No intraleukocytic pigments were observed.

There was no evidence of skin or subcutaneous tissue necrosis in biopsies of severe and uncomplicated malaria.

Intradermal blood smear (Table 2)

Intradermal thick smear : Comparing severe malaria in the first skin squeezing with the second

Table 2
Histologic findings of skin in malaria patients during positive and negative parasitemia by finger prick.

	Positive parasitemia		Negative parasitemia	
	Severe malaria (n = 10)	Uncomplicated malaria (n = 10)	Severe malaria (n = 10)	Uncomplicated malaria (n = 10)
Diapedesis in superficial dermis	1 (10%)	2 (20%)	0 (0%)	0 (0%)
deep dermis	5 (50%)	5 (50%)	9 (90%)	5 (50%)
SFT	10 (100%)	9 (90%)	10 (100%)	8 (80%)
Presence of PRBC	10 (100%)	9 (90%)	8 (80%)	5 (50%)
% PRBC sequestered in vessels of				
superficial dermis	0.7 ± 0.95	0	0	0
deep dermis	5.7 ± 6.60	0.9 ± 2.51	0.4 ± 0.7	0
SFT	67.8 ± 25.66	4.5 ± 2.84	2.8 ± 2.35	0.9 ± 1.10
Sequestration of vessels in				
superficial dermis	2 (20%)	0 (0%)	0 (0%)	0 (0%)
deep dermis	6 (60%)	1 (10%)	1 (10%)	0
SFT	9 (90%)	4 (40%)	5 (50%)	1 (10%)
Tightness of packing of RBC (scored as 0-2+) in vessels of				
superficial dermis	0.4 ± 0.7	0	0	0
deep dermis	1 ± 0.82	0.4 ± 0.52	0.5 ± 0.53	0.1 ± 0.32
SFT	1.8 ± 0.63	1.1 ± 0.74	1.2 ± 0.79	0.7 ± 0.67
% vessels in deep dermis with				
2+ packing	2.2 ± 4.73	0.5 ± 1.27	0	0
1+ packing	1.2 ± 1.88	0.2 ± 0.42	0.6 ± 0.7	0.1 ± 0.32
0+ packing	0	0	0	0
% vessels in SFT with				
2+ packing	63.2 ± 31.37	1.7 ± 2.75	2.1 ± 3.28	0.2 ± 0.63
1+ packing	0	1.4 ± 1.71	0.9 ± 1.1	0.7 ± 0.82
0 packing	0	0.1 ± 0.32	0	0
% vessels containing PRBC in				
superficial dermis	0.6 ± 0.84	0	0	0
deep dermis	6.4 ± 9.31	0.1 ± 0.32	0.4 ± 0.7	0
SFT	71.10 ± 27.71	4 ± 3.09	2.8 ± 2.35	1 ± 1.33
Presence of leukocyte	10 (100%)	10 (10%)	9 (90%)	9 (90%)
platelet	7 (70%)	8 (80%)	8 (80%)	9 (90%)
gametocyte	4 (40%)	5 (50%)	5 (50%)	5 (50%)
Endothelial cells ingesting malarial pigment	10 (100%)	9 (90%)	9 (90%)	10 (100%)
Intraleukocytic pigment (WBC ingesting malaria pigments)	10 (100%)	9 (90%)	9 (90%)	10 (100%)
Thick smear - asexual form	9 (90%)	10 (100%)	6 (60%)	2 (20%)
- gametocyte	8 (80%)	6 (60%)	5 (50%)	6 (60%)
- intraleukocytic pigment	10 (100%)	9 (90%)	8 (80%)	8 (80%)
Thin smear - asexual form	9 (90%)	10 (100%)	5 (50%)	2 (20%)
- gametocyte	6 (60%)	5 (50%)	5 (50%)	5 (50%)
- intraleukocytic pigment	9 (90%)	9 (90%)	9 (90%)	9 (90%)

SFT=subcutaneous fatty tissue; PRBC=parasitized red blood cells; RBC=red blood cells; WBC=white blood cells

skin squeezing, there were no significant differences in the positive findings of asexual forms, gametocytes and intraleukocytic pigments. Asexual forms, gametocytes and intraleukocytic pigments were frequently seen in the first and second skin squeezings of severe malaria. Comparing severe malaria and uncomplicated malaria in the first skin squeezings, there were no significant differences in the positive findings of asexual forms, gametocytes and intraleukocytic pigments. Asexual forms, gametocytes and intraleukocytic pigments were frequently seen in the first skin squeezings of severe malaria and uncomplicated malaria patients. Comparing severe malaria with uncomplicated malaria in the second skin squeezings, there were no significant differences in the positive findings of asexual forms, gametocytes and intraleukocytic pigments. Asexual forms, gametocytes and intraleukocytic pigments were frequently seen in the second skin squeezings of severe malaria and uncomplicated malaria. Comparing findings of the first skin squeezing with the second skin squeezing in uncomplicated malaria, asexual forms were frequently found in the first skin squeezing on admission with positive parasitemia rather than in the second skin squeezing after treatment leading to negative parasitemia ($p = 0.001$).

Intradermal thin smear : Thin smears from intradermal squeezing showed similar findings to thick smears from intradermal squeezing. Comparing severe malaria in the first skin squeezing with the second skin squeezing, there were no significant differences in the positive findings of asexual forms, gametocytes and intraleukocytic pigments. Asexual forms, gametocytes and intraleukocytic pigments were frequently seen in the first and the second skin squeezings of severe malaria. Comparing severe malaria and uncomplicated malaria in the first skin squeezings, there were no significant differences in the positive findings of asexual forms, gametocytes and intraleukocytic pigments. Asexual forms, gametocytes and intraleukocytic pigments were frequently seen in the first skin squeezings of severe malaria and uncomplicated malaria. Comparing severe malaria with uncomplicated malaria in the second squeezings, there were no significant differences in the positive findings of asexual forms, gametocytes and intraleukocytic pigments. Asexual forms, gametocyte and intraleukocytic pigments were frequently seen in the second skin squeezings of severe malaria and uncomplicated malaria. Comparing findings of the first biopsy with the second skin squeezings in uncomplicated malaria, asexual forms were frequently found in the first biopsy on admission with positive parasitemia by finger prick rather than in the second skin squeezing after treatment leading to negative parasitemia by finger prick ($p = 0.001$). Within 6 hours after blood smear by finger prick showed negative parasitemia, asexual forms, gametocytes and intraleukocytic pigments were still frequently found in blood from intradermal squeezing with both intradermal thin and thick blood smears.

The third intradermal squeezing done in one severe malaria patient 14 days after the second skin squeezing showed positive findings of gametocytes in both thin and thick intradermal blood smears.

Immunohistopathology (Figs 3-5)

INFγ, TNFα, IL-1β, IL-2, IL-6, IL-10, GM-CSF, factor 8 were detected by immunofluorescence in different compartments of skin and subcutaneous tissues including stratum corneum, stratum granulosum, stratum spinosum, basal layer, muscle, vessel and collagen. Density varied from 3+ (positive and highest density) to 0 (negative result or negative staining). "3+, 2+, 1+, weakly and 0" scores were weighted as 3, 2, 1, 0.5 and 0 respectively for calculation.

Various densities of immunofluorescence staining were observed in skin and subcutaneous tissues. Comparing mean scores of severe malaria at the first biopsy with those at the second biopsy, there was no significant difference. Comparing mean scores of severe malaria with mean scores of uncomplicated malaria at the first biopsies, there were higher mean scores of TNFα at vessels (2.1 ± 0.99 *vs* 0.45 ± 0.50; $p < 0.001$) and IL-10 at collagen (1.15 ± 0.82 *vs* 0.2 ± 0.42; $p = 0.004$) in severe malaria than in uncomplicated malaria. Comparing mean scores of severe malaria with those of uncomplicated malaria at the second biopsies, there was no significant difference. Comparing mean scores of uncomplicated malaria at the first biopsy with those of the second biopsy, there was also no significant difference. Although blood vessels were histopathologically observed in all biopsy specimens of severe and uncomplicated patients, factor 8 which showed the presence of vessels in skin biopsies was clearly observed in 8 (80%) severe patients in the both first and second biopsies and also in 7 (70%) uncomplicated patients in the both first and second biopsies.

The immunohistologic study of one severe malaria patient was performed for the third time

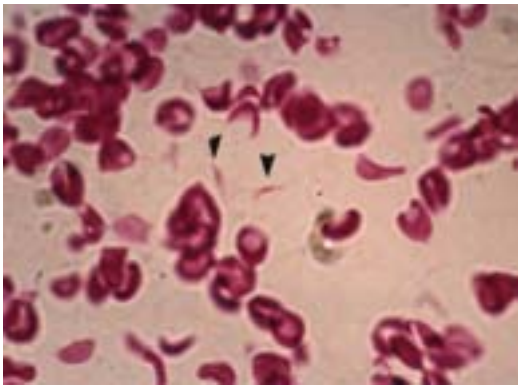

Fig 1–Skin biopsy from a severe malaria patient showing gametocytes (arrowhead) in diapedesis of erythrocytes in dermis (Thomas x 100). Diapedesis was found in the middle of the skin biopsy beyond the biopsy edge and needle puncture site (for xylocain infiltration before skin biopsy). Biopsy was done during positive parasitemia by finger prick.

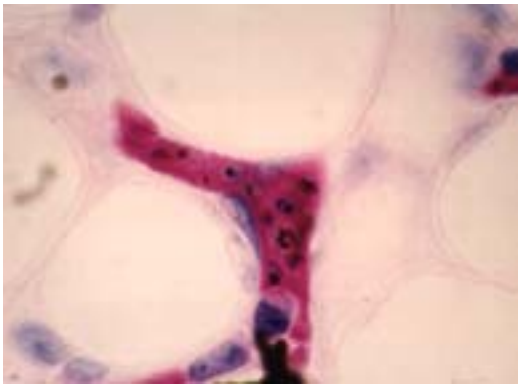

Fig 2–Skin biopsy from a severe malaria patient showing sequestration in a VSFT (Thomas x 100). Biopsy was done during positive parasitemia by finger prick.

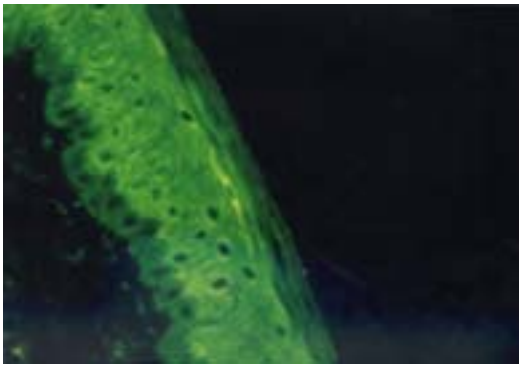

Fig 3–Skin biopsy from a severe malaria patient showing antibody specific to TNFα reacted 3+ at stratum granulosum; 2+ at stratum spinosum; and 1+ at stratum corneum, basal layer and dendritic cells in dermis (Immunofluorescence). Biopsy was done during positive parasitemia by finger prick.

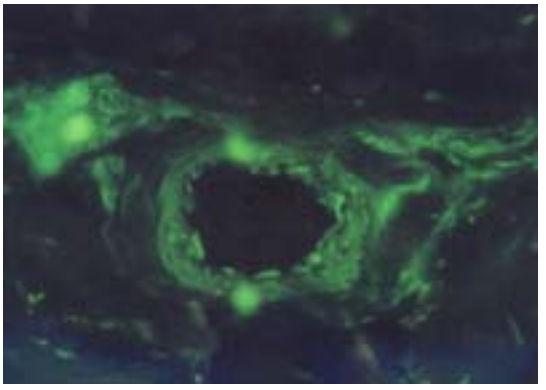

Fig 4–Skin biopsy from a severe malaria patient showing antibody specific to TNFα reacted 1+ with VSFT (Immunofluorescence). Biopsy was done during positive parasitemia by finger prick.

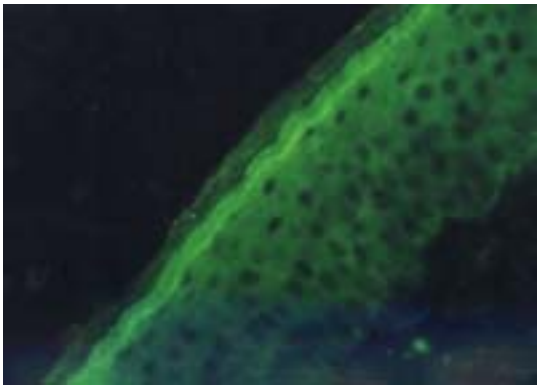

Fig 5–Skin biopsy from a severe malaria patient showing antibody specific to IL-10 reacted 2+ with stratum granulosum; and 1+ with stratum corneum and stratum spinosum (Immunofluorescence). Biopsy was done during positive parasitemia by finger prick.

14 days after the second biopsy because there were still high densities (3+ score) of staining of INFγ (at stratum granulosum, stratum spinosum), TNFα (at stratum corneum, stratum granulosum and stratum spinosum) and IL-6 (at collagen) in the second biopsy. The third biopsy showed high density (3+ score) of staining of INFγ at muscle.

Skin reactions did not show reactivity with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin alone. There was no autofluorescence in the skin sections.

DISCUSSION

In this study, although there were no differences in parasitemias by finger prick between severe and uncomplicated malaria (possibly due to the rather small number of the sample sizes), the disease severity could be ascertained by clinical and laboratory parameters as shown in Table 1. As regards to blood smear on admission, differences in microscopic findings were only of schizonts which were helpful to discriminate severe from uncomplicated malaria only 7 of 10 patients (70%) in the severe group.

Histopathologic findings of skin biopsy showed significantly different findings between severe and uncomplicated malaria on admission with the first biopsy, particularly density of sequestration in VSFT. Within 6 hours of negative parasitemia (after treatment), severe malaria sequestration in SFT was reduced and was similar to sequestration in uncomplicated malaria. With our current knowledge, there is no known explanation for more sequestration in VSFT than in other compartments of the skin biopsy specimen. This finding corresponded to a previous report of correlation between a high sequestration rate in subcutaneous tissue and cerebral microvessels in a rhesus monkey with cerebral malaria (Nakano *et al*, 1996).

Intradermal smears showed similar findings between severe and uncomplicated malaria both at the first and the second skin squeezings. Within 6 hours after negative parasitemia by finger prick, asexual forms, gametocytes and intraleukocytic pigments were seen in intradermal blood thick and thin smears. Thus an intradermal blood smear is more sensitive for detection of parasitemia than a finger prick smear because intradermal blood smear possibly squeezed parasites sequestered in vessels of skin. This showed that negative parasitemia by finger prick is not an absolute, predictive term to be used since a finger prick smear cannot detect sequestered parasites (which are an important part of the pathophysiology of severe malaria). Therefore, in assessment of antimalarial drug efficacy, intradermal smears may be helpful in evaluation of parasitemia in the human body. As intradermal smears were not frequently performed, particularly every 6 hours after negative parasitemia by finger prick, the time in which negative parasitemia by intradermal smear can be detected is not yet known. Only one severe malaria patient was restudied 14 days after second skin squeezing: the intradermal smear was only positive for gametocytes in both thin and thick smears and negative for asexual forms and intradermal leukocyte pigments. The third skin squeezing revealed that the patient had a previous malaria infection because of the presence of gametocytes, and the finding also corresponded with the finger prick smear showing gametocytes in the thick smear.

Several studies of severe/cerebral malaria have shown significant associations between high levels of cytokines in plasma and disease severity. However the half-life of certain cytokines in blood, particularly TNFα, is short, and even significant tissue production may not be reflected accurately by systemic concentrations (Day *et al*, 1999). There is only one report of tissue cytokine in fatal cerebral malaria and the study clearly detected INFγ, TNFα, IL-1β, IL-10 in paraffin-embedded tissues from the brain and the liver but not in skin (Udomsangpetch *et al*, 1997). The possible explanations are small sample size (only two fatal patients) in the previous study and short-life cytokines could not be found during autopsy. In the present study, skin biopsies were done in live patients and immediately processed for immunofluorescence study, thus doing away with the time factor delay before immunofluroescence study.

TNFα at vessels and IL-10 at collagen had significantly higher density in severe malaria than in uncomplicated malaria at the first biopsy. Thus, high levels of TNFα at vessels and IL-10 at collagen may indicate disease severity. The other cytokines showed no significant differences between the two groups. In severe and uncomplicated malaria, there was no significant difference in cytokine density between the first biopsy and the second biopsy, possibly due to the short time between both biopsies (PCT=52.2±13.37 hours and 48.8±12.04 hours in severe and uncomplicated malaria respectively). The parasites were rapidly cleared with artemisinin derivatives. After antimalarial treatment with negative parasitemia by finger prick there was no significant difference in cytokine density between severe and uncomplicated malaria, possibly due to reduced inflammation after treatment. Therefore, the reason for negative staining of the skin in the previous report (Udomsangpetch *et al*, 1997) may be due to the small sample size studied or the time factor delay before immunufluorescence study, rather than true negative immunofluorescence staining of the skin.

One severe patient with negative parasitemia by finger prick was restudied (finger prick, intra-

dermal smear and skin biopsy) 14 days after the second study. The result showed that antibodies to INFγ and factor 8 still gave high reactions (score=3+) even 14 days after treatment. The function of INFγ in immunologic enhancement and protection against malaria may be explained. However, it is as yet unknown when all cytokine reactions become negative after antimalarial treatment.

The presence of cytokines in the tissues studied was determined to assess the relationship with histopathogenesis in falciparum malaria. The finding that TNFα and IL-10 were readily detectable in the skin biopsy suggests that these cytokines may play an important role in the major histopathologic changes observed in many previous studies (Spitz, 1946; Riganti *et al*, 1990; Boonpucknavig *et al*, 1990).

Various patterns of cytokines found in tissues may indicate differences in the level of endothelial cell activation by sequestered parasites. Recent reports showed that high levels of plasma TNFα and IFNγ in falciparum malaria appear to be related to the severity of disease (Kwiatkowski *et al*, 1990; Harpaz *et al*, 1992). TNFα induces neutrophil adherence and accumulation in small vessels (Liu *et al*, 1994), and infiltration of neutrophils into inflammatory tissues has been shown to be associated with an increase in tissue levels of cytokines (Lloyd and Oppenheim, 1992; Dinarello and Wolff, 1993). IL-10 (Ho *et al*, 1995) has been shown to inhibit the release of TNFα, and similarly IFNγ can inhibit the production of IL-10 by monocytes (Chomarat *et al*, 1993). This may explain why TNFα was not detected in some cases and why IL-10 was not detected in some other cases. However, cytokine production can be the result of an inflammatory response, which might then alternatively explain the difference in cytokine patterns found in the patients. In this study, there were no normal control skin biopsies for immunofluorescence staining; however the results of positve and negative stainings were true positive and true negative stainings respectively because skin reactions did not show reactivity with FICT-conjugated anti-rabbit immunoglobulin alone and also there was no autofluorescence in the skin sections. Although the implications of these two cytokines in severe malaria patients are not clear, this study signifies the clinical relevance of cytokines in the histopathogenesis of disease severity of falciparum malaria and emphasizes the need for further investigation.

In conclusion, intradermal blood smear provides greather sensitivity for parasite detection 6 hours after a negative finger prick blood smear but cannot indicate the prognosis of disease severity. % PRBC in VSFT, tightness of packing of RBC in VSFT, % VSFT with 2+ packing and % vessels containing PRBC in SFT were significantly associated with disease severity. High sequestration is frequently found with high density in VSFT in severe malaria. INFγ, TNFα, IL-1β, IL-2, IL-6, IL-10, GM-CSF cytokines were found in skin and subcutaneous tissue of malaria with various densities; however TNFα at vessels and IL-10 at collagen is significantly higher in severe malaria and may indicate prognostic severity.

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