A QUANTITATIVE ULTRASTRUCTURAL STUDY OF THE LIVER AND THE SPLEEN IN FATAL FALCIPARUM MALARIA

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Abstract. We performed a retrospective study of 25 patients who died of severe falciparum malaria in Thailand and Vietnam using electron microscopy. The aims of the study were: to determine if there was any significant association between parasitized red blood cells (PRBC) sequestered in liver and spleen and particular pre-mortem clinical complications, and to compare the degree of parasite load between the liver and spleen within the same patients. PRBC sequestrations in each organ were compared with the pre-mortem parasitemia, to calculate the sequestration index (S.I.). The S.I. showed that the degree of PRBC sequestration in the spleen was higher than the liver (S.I. median = 3.13, 0.87, respectively) ($p < 0.05$). The results of quantitative ultrastructural study showed a significantly high parasite load in the liver of patients with jaundice, hepatomegaly and liver enzyme elevation ($p < 0.05$). We found a significant correlation between PRBC sequestration in the liver and a high serum bilirubin level, a high aspartate aminotransferase (AST) level and an increase in the size of the liver (Spearman's correlation coefficient = 0.688, 0.572, 0.736, respectively). Furthermore, a higher parasite load was found in the liver of patients with acute renal failure (ARF) compared to patients without ARF ($p<0.05$). These findings suggest that PRBC sequestration in the liver is quantitatively associated with pre-mortem hepatic dysfunction and renal impairment. There was no significant difference between splenomegaly and PRBC sequestration. The size of a palpable spleen was not correlated with parasite load in the spleen. When ultrastructural features were compared between the two reticuloendothelial organs, we found that the spleen had more PRBC and phagocytes than the liver. The spleen of non-cerebral malaria (NCM) patients had more phagocytes than cerebral malaria (CM) patients. This observation reveals that the spleen plays a major role in malaria parasite clearance, and is associated with host defence mechanisms against malaria.

INTRODUCTION

Severe falciparum malaria can cause many clinical manifestations, which involve several organs (White and Ho, 1992). These include cerebral malaria (CM), acute renal failure (ARF), acute respiratory distress syndrome (ARDS), pulmonary edema, severe hemolysis and shock. Sequestration of PRBC in specific organs has been proposed as an important pathophysiological factor in clinical patterns of severe malaria. CM accounts for many of the deaths in severe malaria infected patients. Most previous studies have tended to concentrate on the pathophysiology of changes in the brain (Macpherson *et al*, 1985; Pongponratn *et al*, 1991.) The degree of sequestration of parasitized red blood cells (PRBC) in cerebral microvessels has been found to be associated with pre-mortem coma (Pongponratn *et al*, 2003). There has been no reported study of the ultrastructure focusing on the association between the sequestration of PRBC in other organs and the particular clinical

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complications. Several studies have reported abnormal liver and spleen function during malaria, indicated by hepatomegaly (Sowunmi *et al*, 2001), splenomegaly (Looareesuwan *et al,* 1987) and liver enzyme elevation (Premaratna *et al*, 2001; Kochar *et al*, 2003). Jaundice has often been reported in patients with cerebral malaria, hyperparasitemia, acute renal failure and shock (Wilairatana *et al*, 1994). The pathology that causes hepatic dysfunction and the role of the spleen in *Plasmodium falciparum* malaria in humans is still unclear. The main objectives of this study were to demonstrate PRBC sequestration in reticuloendothelial organs, including the liver and spleen, and to determine whether premortem clinical complications, such as jaundice and hepatosplenomegaly were associated with PRBC sequestration in these organs. The other objectives were to correlate the clinical complications with pathological findings in fatal falciparum malaria and to examine the interactions between PRBC and host cells, including endothelial cells, leukocytes and platelets.

MATERIALS AND METHODS

Patients

Specimens were collected from patients who died of severe falciparum malaria in Thailand and Vietnam from 1987 to 2001. A full autopsy was performed on these patients with routine pathological examinations. When autopsy was not possible, percutaneous needle biopsy was performed on some cases. Informed consent for autopsy and tissue necropsy were obtained from the patients or their accompanying relatives. Ultrastructural studies were done without the knowledge of clinical information until the end of the statistical analysis.

Ultrastructural studies

The tissues were cut and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. Fixed tissues were subsequently washed three times with 0.1 M phosphate buffer at pH 7.4, post fixed with 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.4, at 4ºC for 1 hour, dehydrated in a graded ethanol series, infiltrated with propylene oxide, and embedded in Epon 812 or spurr's epoxy resin. Ultrathin sections were cut with glass knives, mounted on copper grids (200-mesh square), stained with 2% uranyl acetate and Reynold's lead citrate prior to examine in a transmission electron microscope (Hitachi H-7000 model). Qualitative and quantitative ultrastructural analysis was performed on ten grid squares of the copper grids depending on the size of the tissues.

Qualitative ultrastructural analysis

The presence or absence of sequestered PRBC, leukocytes, fibrin thrombi, platelets and endothelial cells changes was recorded. Electron micrographs were taken of relevant areas.

Quantitative ultrastructural analysis

The number of NRBC and PRBC, which were identified as knob positive PRBC(K^+PRBC), knob negative PRBC (K^-PRBC) and schizonts (defined as multisegmented parasites with more than three nuclei per PRBC) were counted. Only macrophages, white blood cells or endothelial cells that showed phagocytosed NRBC, PRBC or malarial pigments were counted as phagocytes. All counting parameters were recorded in terms of the number counted per grid square.

The parasite load in each organ were calculated and expressed as a sequestration index (S.I.). The S.I. has been previously described by Silamut *et al* (1999) as a way to estimate the difference between PRBC accumulation in a tissue vascular bed, compared to the peripheral blood parasitemia. The S.I. was calculated from the ratio of the percentage of PRBC in a tissue vascular bed to the percentage of PRBC in the peripheral blood.

Statistical methods

Data were analyzed by SPSS for Windows version 11.0. To compare medians between the different clinical groups, we used the Mann-Whitney *U* test. For comparison of the percentages of cases with and without PRBC between different clinical groups. The chi-square test or Fisher's exact test were used. To compare the parasite load between the liver and spleen within the same patients, the Wilcoxon signed rank test was used. We used Spearman's correlation coefficient to explain the correlation between PRBC sequestration in the liver and the level of serum bilirubin or liver enzymes.

RESULTS

Summary of clinical data

The clinical summaries of the malaria cases are shown in Table 1. There were 25 patients included in this study. Of these, 19 liver tissues and 14 spleen tissues were collected. Only 8 patients had tissue samples collected from both organs were compared.

Qualitative ultrastructural analysis

Degenerative post-mortem changes were seen. However despite these changes, the presence of obvious pathological processes was observed. Ultrastructural features of the spleen showed congestion of splenic sinusoids and the splenic artery with normal and PRBC (Fig 1A). Splenic pitting of PRBC was commonly seen (Fig 1B and 1C). Ultrastructural features of the liver showed less congestion compared to the spleen. Hepatic sinusoids were filled with NRBC, PRBC, schizonts and phagocytes. In these patients, all parasite stages included K^-PRBC, K^+PRBC and schizonts. Occasionally gametocytes were found; many of them had an irregular shape, characterized by distortion and elongation of cytoplasmic processes (Fig 1D). Platelets were occasionally found. It was rare to find fibrin deposition (Fig 1E). Phagocytosis of RBC, PRBC and malarial pigment was always found in both reticuloendothelial organs and more predominantly was associated with mononuclear cells (MNC) (Fig 1F). Reactive Kupffer's cells containing PRBC, RBC or malarial pigment were commonly seen (Fig 2A). There was clear evidence of PRBC adhering to sinusoidal endothelial cells observed in the liver and spleen of this study (Fig 2B). Minimal changes in the endothelial cells were seen.

Quantitative ultrastructural analysis

The spleen. To determine whether the pathological features of the spleen were associated with the complications of falciparum malaria, the counting parameters of the spleen were compared among the different clinical groups. Comparative quantitation of the counting parameters per grid square of the spleen and in the different clinical groups is shown in Table 2. Statistical analysis showed no differences in the number of grid squares counted between the patient groups. There was no significant difference in most of the counting parameters examined of the spleen in the clinical groups except for the number of phagocytes, which was significantly higher in the NCM and jaundice patients compared to the CM and patients with no jaundice (p=0.024, p=0.030, respectively).

The liver. Comparative quantitation of the counting parameters examined in the liver among the different clinical groups is shown in Table 3. Statistical analysis showed no differences in the number of grid squares counted in the patient groups. There were no significant differences in most of the parameters examined in the livers of CM patients compared to NCM patients. Comparing patients with and without jaundice, the following parameters were significantly higher in patients with jaundice compared to patients without jaundice: the percentage of PRBC (p=0.028), S.I. of PRBC (p=0.011) and S.I. of K^- PRBC (p=0.008). The chi-square test and Fisher's exact test were used to compare percentages, and no significant associations were found between patients with jaundice and the percentage of cases with PRBC and K^-PRBC (p=0.005 and p=0.007, respectively).

The clinical and laboratory findings of malaria patients are given in Table 4. Blood chemistry showed an elevation in serum bilirubin (mean 29.28 mg/dl; range 4.22-175 mg/dl) in patients with jaundice. There was a significant correlation between the S.I. of PRBC and the level of serum bilirubin, including the total and direct bilirubin (Spearman's correlation coefficient=0.688, 0.617; p=0.002, 0.006, respectively; Table 3).

Significantly higher percentages of PRBC and K^-PRBC were found in patients with liver enzyme elevation compared to those without liver enzyme elevation (p=0.033 and p=0.042, respectively; Table 3). There was a significant correlation between the percentage of PRBC in the liver and the level of aspartate transaminase (AST) (mean = 88.23 U/ml; range 22-291 U/ml; Spearman's correlation coefficient = 0.572; p=0.026; Table 3).

As with others (Nacher *et al*, 2001b), we considered a palpable liver as a reflection of hepatomegaly. Because a palpable liver is not the best measure of hepatomegaly, we defined

Table 1
Clinical details of the malaria cases (n = 25).

Case number	Age (yrs)	Sex	Drug	Time to death (hrs)	Admission parasite count/μl	Last parasite count/ μl (%)	Diag	EM liver parasite count (%)	EM spleen parasite count (%)	Other complications of disease
1	25	M	A	13.5	967,120	815,500 (29.51)	CM	45.61	28.14	ARF, ARDS, Hep, J, S, Hypogly
2	16	M	A	1.5	NA	NA	CM	NA	2.48	ARF, Pulm Ed, C
3	61	M	A	296.5	3,692,640	1,195,335 (30.7)	CM	6.49	NA	ARF, Pulm Ed, HyperP
4	22	M	A	17	189,028	8,540 (0.2)	CM	0	NA	ARDS
5	55	F	A	77	57,250	160 (0.1)	CM	1.11	NA	ARF, ARDS, Hep, J, Hypogly
6	38	F	A	80	1,271,160	80 (0.3)	CM	0	1.39	None
7	40	M	Q	38	58,696	455,400 (13.95)	CM	9.61	NA	None
8	19	F	Q	53	215,176	29,601 (1.2)	NCM	4.5	NA	Hep, J, An, DIC, sepsis
9	20	M	Q	3	1,360,128	1,360,128 (44.8)	NCM	13.5	NA	J, An, HyperP
10	45	M	Q	53.5	160,454	0	CM	0.87	2.79	ARF, J, S
11	67	M	Q	24	3,030	12,434 (0.3)	CM	0.69	NA	None
12	26	F	Q	8	444,624	444,624 (17.5)	CM	6.27	10.08	ARF, J, S
13	19	M	Q	16	72,848	72,848 (2.87)	CM	17.8	30.64	ARF, J, An, Acidosis
14	27	M	Q	81	1,244,947	6,426 (0.2)	CM	1.44	7.17	J
15	57	M	Q	57	179,503	16,192 (0.34)	CM	8.58	NA	Hep, J, S
16	26	M	Q	31	66,882	564,572 (18.73)	CM	8.11	NA	ARF, J, S, HyperP
17	63	M	A	23	84,403	560 (0.015)	CM	NA	24.37	Hep, J, S,C
18	34	M	Q	4	805,912	805,912 (22.3)	NCM	NA	45.5	J, C
19	28	M	A	4	546,360	958,830 (34.9)	CM	NA	22.82	Hep, S, HyperP
20	28	M	Q	44	619,208	128,865 (5.4)	NCM	NA	16.88	An, S, C
21	28	M	Q	95.75	45,342	0	CM	20.95	2.18	Hep, J, An, Hypogly
22	34	M	Q	6.6	150,720	53,882 (3.3)	CM	33.22	23.29	ARF, J, S, An
23	22	F	Q	27.3	802,584	56,520 (1.8)	NCM	NA	20.65	Pulm Ed , Hep, J, S,HyperP
24	56	F	A	4.66	21,101	117,096 (4.7)	NCM	0	NA	None
25	28	M	A	18.5	629,256	148,836 (7.9)	NCM	3.98	NA	Pulm Ed , J, An, Hypogly

A = artemisinin derivatives, An = anemia, ARDS = acute respiratory distress syndrome, ARF = acute renal failure, C = coma, CM = cerebral malaria, Hep = hepatomegaly, HyperP = hyperparasitemia, Hypogly = hypoglycemia, J = jaundice, NA = not available, Pulm Ed = pulmonary edema, NCM = non-cerebral malaria, Q = quinine, S = shock

Table 2
Comparative quantitation of pathological features of the spleen between different clinical groups.
Significant p-values ($p<0.05$) are marked in bold.

Parameters	CM (n=11)	NCM (n= 3)	p	ARF (n=6)	NARF (n= 8)	p	Jaundice (n=11)	No jaundice (n= 2)	p
Grid squares counted	10 (6-13)	10 (10-11)	0.933	10 (9-11)	10 (6-13)	0.678	10 (9-13)	8 (6-10)	0.117
RBC	131.89 (71-200.30)	166.80 (124.20-175.09)	0.312	123.39 (98.18-200.30)	151.52 (71-175.09)	0.439	138.33 (98.18-200.30)	117.85 (71-164.70)	0.43
PRBC	14.78 (1-88.50)	43.40 (35.55-103.70)	0.312	29.89 (2.80-88.50)	39.48 (1-103.70)	0.796	43.40 (2.82-103.70)	24.85 (1-48.70)	0.554
% PRBC	10.08 (1.39-30.64)	20.65 (16.88-45.50)	0.312	16.69 (2.48-30.64)	18.77 (1.39-45.50)	0.699	20.65 (2.18-45.50)	12.11 (1.39-22.82)	0.324
S.I. (PRBC)	3.71 (0.58-1624.67)	3.13 (2.04-11.47)	0.866	2.79 (0.58-10.68)	3.88 (0.65-1624.67)	0.306	3.13 (0.58-1624.67)	2.64 (0.65-4.63)	0.554
K^+PRBC	4.08 (0-70.20)	32.60 (5.27-71.10)	0.102	7.66 (0.30-70.20)	13.19 (0-71.10)	0.897	13.20 (0.08-71.10)	10.55 (0-21.10)	0.324
% K^+PRBC	3.02 (0-24.31)	15.51 (2.50-31.20)	0.139	5.12 (0.27-24.31)	6.46 (0-31.20)	0.897	8.26 (0.06-31.20)	4.95 (0-9.89)	0.324
S.I. (K^+PRBC)	0.28 (0-808)	1.40 (0.46-8.62)	0.483	1.13 (0-8.47)	0.93 (0-808)	0.605	1.98 (0.06-808)	0.14 (0-0.28)	0.093
K^-PRBC	12 (0.82-31.80)	29.64 (9.90-31.40)	0.186	14.60 (0.82-31.80)	15.65 (1-31.40)	0.699	17.20 (0.82-31.80)	11.20 (1-21.40)	0.554
% K^-PRBC	5.96 (0.81-19.89)	13.78 (4.71-14.07)	0.186	7.07 (0.81-19.89)	7.37 (1.39-14.07)	0.796	8.18 (0.81-19.89)	5.71 (1.39-10.03)	0.554
S.I. K^-PRBC	2.08 (0-780.67)	2.61 (0.62-2.62)	0.938	0.74 (0-2.63)	2.62 (0.29-780.67)	0.121	2.12 (0.47-780.67)	2.46 (0.29-4.63)	0.693
Phagocyte	1.23 (0.60-3.36)	4.55 (3.20-4.90)	0.024	2.35 (0.60-3.36)	1.49 (0.80-4.90)	1.000	3.10 (0.90-4.90)	0.82 (0.80-0.83)	0.03

ARF = patients with acute renal failure, CM = patients with cerebral malaria, NARF = patients without acute renal failure, NCM = patients without cerebral malaria

hepatomegaly as when patients had a liver palpable >2 cm below the costal margin. Using this definition, we found a significantly higher S.I. PRBC and S.I. K^-PRBC in patients who had hepatomegaly ($p=0.009$ and $p=0.001$, respectively; Table 3). There was a significant correlation between the size of the liver and the S.I. of PRBC and S.I. of K^-PRBC (Spearman's correlation coefficient = 0.736 ($p=0.0001$) and 0.736 ($p=0.0001$), respectively; Table 3).

Comparison of pathological features between the liver and spleen

For the patients with specimens collected from both liver and spleen, Wilcoxon signed ranks test was used to compare the parameters examined in both organs. A comparison of parameters in the liver and spleen is given in Table 5. The results show that the amount of uninfected RBC and PRBC, including K^+PRBC, K^-PRBC, and schizonts, was significantly higher in the spleens than in the livers of the fatal cases ($p=0.012$, $p=0.012$, $p=0.018$, $p=0.012$, $p=0.046$, respectively). There were significantly more phagocytes ($p=0.012$) in the spleens than the livers of the matched cases.

DISCUSSION

In falciparum malaria, obstruction of small vessels resulting from adhesion of parasitized red blood cells to endothelial cells has been reported in several studies (Macpherson *et al*, 1985; Aikawa *et al,* 1990; Pongponratn *et al*, 1991). This phenomenon has been related to the pathogenesis of the lesions, mainly in the brain. Previous quantitative studies of sequestration in different organs from fatal cases confirmed that the sequestration of PRBC in cerebral microvasculature was associated significantly with clinical cerebral malaria (Macpherson *et al,* 1985; Riganti *et al*, 1990; Turner, 1997, Pongponratn *et al*, 2003). A study by Pongponratn *et al* (2003) confirmed the basic ultrastructural findings that are common to all pathological descriptions of cerebral malaria. This includes the presence of large numbers of PRBC in the microvasculature (sequestration) and to a lesser extent margination of infected red cells in medium and large sized vessels.

Table 3
Comparative quantitation of pathological features of the liver between different clinical groups.
Significant p-values (p<0.05) are marked in bold.

Parameters	CM (n=15)	NCM (n= 4)	p	Hepatome-galy (n=5)	No hepatome-galy (n=13)	p	Jaundice (n=13)	No jaundice (n= 6)	p	Enz. elevation (n= 16)	No enz elevation (n= 3)	p
Grid squares counted	13 (6-21)	11.50 (11-13)	0.685	14 (11-15)	11.5 (6-21)	0.302	12 (6-21)	12 (10-13)	0.328	12.5 (10-21)	11 (6-13)	0.257
RBC	10.60 (2-56.80)	10.49 (2.15 -30.64)	0.841	6.21 (2-14.5)	16.62 (2.15-56.80)	0.116	7.64 (2-56.80)	15.26 (2.15-36.85)	0.792	7.56 (2-56.80)	21.46 (19.91-37.67)	0.094
PRBC	0.53 (0-4.73)	0.82 (0-2.08)	0.764	0.53 (0.07-4.73)	0.89 (0-3.92)	0.745	1.36 (0.07-4.73)	0.08 (0-3.92)	0.065	1.32 (0-4.73)	0.15 (0-0.33)	0.093
% PRBC [d]	6.49 (0-45.61)	4.24 (0-13.50)	0.548	8.56 (1.14-45.61)	5.13 (0-33.22)	0.194	8.11 (0.87-45.61)	0.35 (0-9.61)	0.028	7.30 (0-45.61)	0.69 (0-0.87)	0.033
S.I. (PRBC) [b c e]	1.55 (0-25.22)	0.40 (0-3.75)	0.229	11.15 (1.55-25.22)	0.470 (0-10.07)	0.009	3.75 (0.30-25.22)	0.11 (0-2.31)	0.011	1.12 (0-25.22)	0.87 (0-2.31)	0.502
Number (%) with PRBC [a]	13 (86.7)	3 (75)	0.57	5 (100)	11 (78.6)	0.259	13 (100)	3 (50)	0.005	14 (87.5)	2 (66.7)	0.364
K^+PRBC	0 (0-1.67)	0 (0-0)	0.106	0 (0-0.93)	0 (0-1.67)	0.957	0 (0-1.67)	0 (0-0.15)	0.171	0 (0-1.67)	0 (0-0.15)	0.698
% K^+PRBC	0 (0-29.35)	0 (0-0)	0.106	0 (0-5.86)	0 (0-29.35)	0.873	0 (0-29.35)	0 (0-0.69)	0.171	0 (0-29.35)	0 (0-0.69)	0.698
S.I. (K^+PRBC)	0 (0-17.25)	0 (0-0)	0.106	0 (0-17.25)	0 (0-8.89)	0.789	0 (0-17.25)	0 (0-2.31)	0.244	0 (0-17.25)	0 (0-2.31)	0.897
Number (%) with K^+ PRBC [a]	7 (46.7)	0 (0)	0.086	2 (44)	5 (35.7)	0.865	6 (46.2)	1 (16.7)	0.216	6 (37.5)	1 (33.3)	0.891
K^-PRBC	0.33 (0-3.92)	0.82 (0-2)	0.687	0.40 (0.07-3.91)	0.31 (0-3.92)	0.513	0.40 (0-3.91)	0 (0-3.92)	0.184	0.42 (0-3.92)	0 (0-0.33)	0.091
% K^-PRBC	2.71 (0-37.70)	4.24 (0-12.98)	0.614	4.50 (1.11-37.70)	2.45 (0-12.98)	0.135	3.19 (0-37.70)	0 (0-9.61)	0.133	3.59 (0-37.70)	0 (0-0.87)	0.042
S.I. K^-PRBC [f]	0.69 (0-15.80)	0.39 (0-3.75)	0.762	7.97 (1.28-15.80)	0.25 (0-1.11)	0.001	0.91 (0-15.80)	0 (0-0.69)	0.008	0.59 (0-15.80)	0 (0-0.87)	0.176
Number (%) with K^- PRBC [a]	11 (73.3)	3 (75)	0.946	5 (100)	9 (64.3)	0.12	12 (92.3)	2 (33.3)	0.007	13 (81.3)	1 (33.3)	0.084

ARF = patients with acute renal failure, CM = patients with cerebral malaria, Enz. elevation = patients with liver enzyme elevation, NARF = patients without acute renal failure, NCM = patients without cerebral malaria, No enz. elevation = patients without liver enzyme elevation

[a] Chi-square test or Fisher's exact test

For association with serum bilirubin (TB=total bilirubin, DB=direct bilirubin), AST (Aspartate aminotranferase) and size of palpable liver , Spearman's correlation coefficient (r) was calculated and significant p-value are given below:

[b] r = 0.688, p = 0.002 (TB), [c] r = 0.617, p = 0.006 (DB), [d] r = 0.572, p = 0.026 (AST), [e] r = 0.736, p = 0.0001 (palpable liver), [f] r = 0.736, p = 0.0001 (palpable liver)

Table 4
Clinical and laboratory findings in malaria patients (n = 19).

Case number	Palpable liver (cm)	Palpable spleen (cm)	TB (mg/dl)	DB (mg/dl)	AST (IU/ml)	ALT (IU/ml)	ALP (IU/ml)
1	3	1	8.05	4.5	291	75	41.4
2	NA	NA	NA	NA	NA	NA	NA
3	0	0	2.95	1.4	193	52	75.3
4	0	0	2.9	0.4	47	20	20.1
5	3	0	34.39	20.8	102	42	56.6
6	0	0	2.02	0.77	64	38	101
7	0	0	3.05	1.02	58.5	12	46
8	3	0	2.72	1.15	55	10.75	30
9	0	0	36.2	20.5	92	36	46
10	2	0	14.54	6.8	25	22	24
11	0	0	0.38	0.2	22	7	44
12	1	1	9.35	4.98	110	78	18
13	1	1	32.6	18.1	66	34	10
14	2	1	4.22	1.45	61	51	19
15	3	0	175	44	73	42	55
16	2	0	4.4	1.78	64	61	52
21	4	0	11.8	6	250	353	NA
22	0.5	0.5	4.4	1.6	140	86	NA
25	0	0.5	16.4	11.8	320	417	NA

ALP = Alkaline phosphatase, ALT = Alanine aminotransferase, AST = Aspartate amino- transferase, DB = Direct bilirubin, TB = Total bilirubin

Table 5
Comparison of parameters between the liver and spleen; matched cases (n=8). Significant p-values ($p<0.05$) are marked in bold.

Parameters	Liver	Spleen	p-value
RBC	6.56 (2-56.8)	128.68 (71-200.3)	0.012
PRBC	1.08 (0-4.73)	12.24 (1-88.5)	0.012
% PRBC	1.08 (0-4.73)	12.24 (1-88.5)	0.779
S.I. (PRBC)	3.88 (0-20.95)	3.71 (0.58-35.85)	0.484
K^+PRBC	0.05 (0-1.67)	3.09 (0-70.2)	0.018
% K^+PRBC	0.09 (0-29.35)	2.5 (0-24.31)	0.237
S.I. (K^+PRBC)	0.01 (0-8.89)	1.13 (0-15.1)	0.176
K^-PRBC	0.31 (0-3.91)	8.81 (0.82-31.8)	0.012
% K^-PRBC	3.09 (0-37.7)	5.06 (0.81-19.89)	0.779
S.I. K^-PRBC	0.89 (0-15.8)	2.1 (0.47-20.8)	0.327
Phagocyte	0 (0-0.33)	1.68 (0.83-3.36)	0.012

Previous studies have tended to concentrate on deaths from cerebral malaria and examine pathological changes in the brain. It has also been reported that other complications, such as pulmonary edema, renal failure, jaundice and hepatosplenomegaly, are as common as coma in this patient population (Gachot *et al*, 1995; Sitprija *et al*, 1996; WHO, 2000).

The liver is the first organ involved in malaria infection, as parasite reproduction following infective mosquito bite takes place here (Barnwell, 2001). Hepatic involvement in a

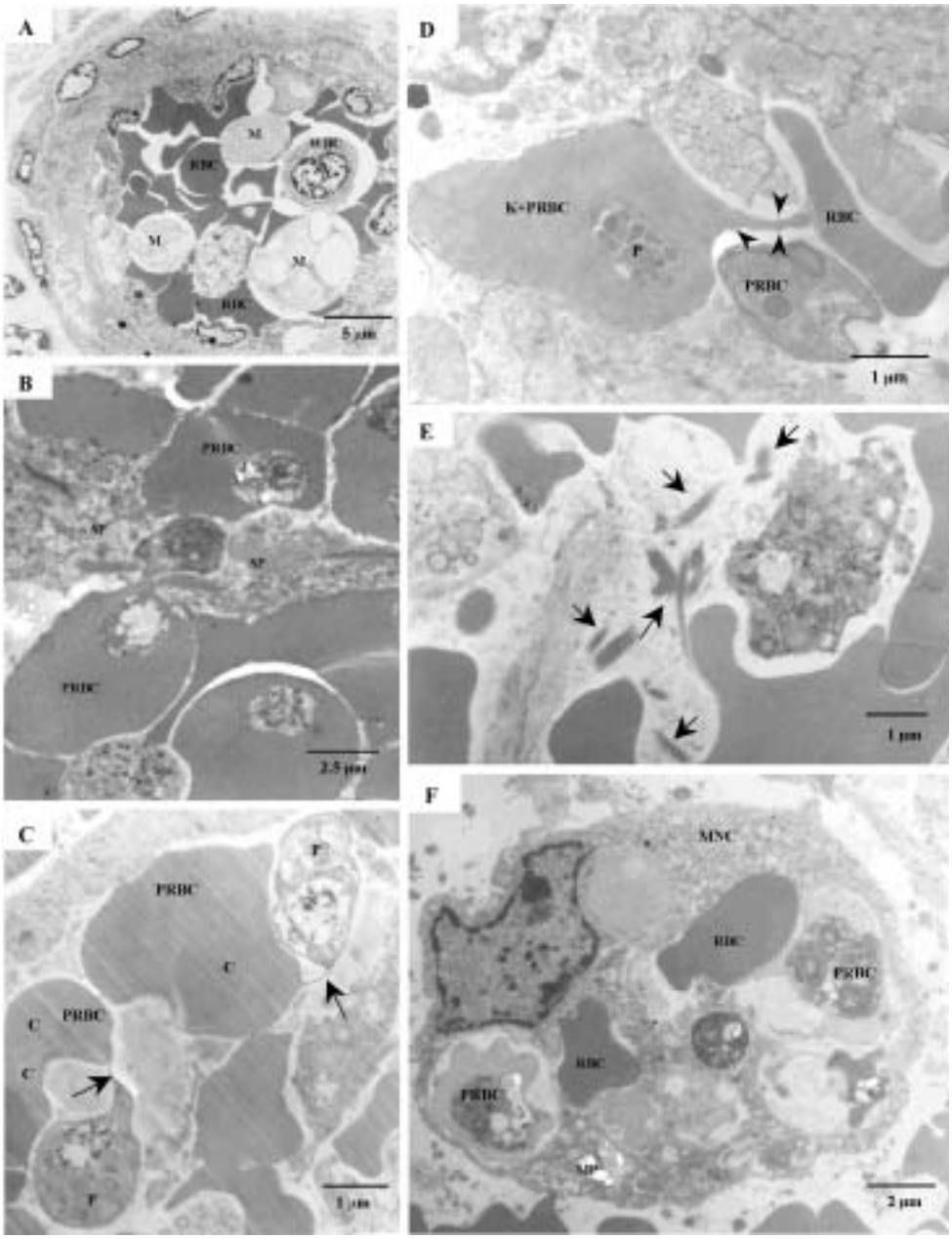

Fig 1A–A splenic artery is filled with RBC, WBC, and some lysed materials (M). n = nucleus of endothelial cells.

Fig 1B–Pitting of PRBC by splenic cell (SP) is commonly seen in the spleen of malaria patients.

Fig 1C–Evidence of splenic pitting is shown. One part of PRBC which contain only intraerythrocytic parasites (P) is linked to the other part by very thin process (arrows). The other part of PRBC contains only cytoplasmic clefts (C).

Fig 1D–A K^+PRBC showing distortion and elongation of cytoplasmic process is seen. Numerous knobs (arrowheads) are shown on its surface membrane.

Fig 1E–Fibrin formation (arrows) in splenic sinusoids was seen in only one fatal case.

Fig 1F–A mononuclear cell (MNC) in splenic sinusoid is seen phagocytosed with RBC, PRBC and malarial pigment (MP) in its cytoplasm.

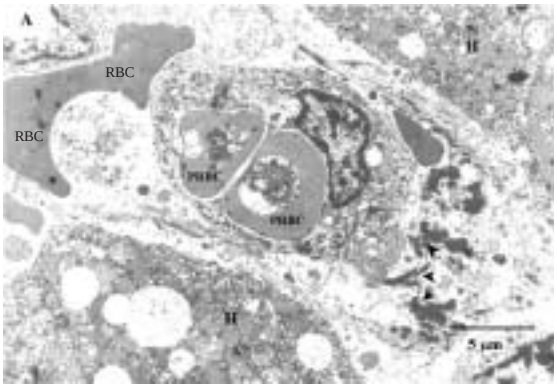

Fig 2A–A reactive Kupffer's cell containing two PRBC is seen in a hepatic sinusoid. Fibrin formation (arrowheads) is also found in this case. n = nucleus of Kupffer's cell.

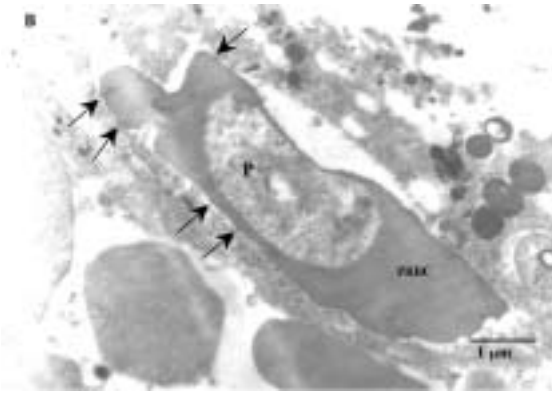

Fig 2B–Evidence of cytoadherence of PRBC in the hepatic sinusoid is shown. Electron dense knobs (arrows) are seen at the point of contact. P= parasite.

significant proportion of malaria patients is based on nonspecific diagnostic tests, like hepatomegaly and jaundice (Ramachandran and Perera, 1976). Previous studies have reported that hepatic dysfunction is associated with falciparum malaria, and jaundice is associated with cerebral malaria, hyperparasitemia, renal failure and shock (Wilairatana *et al,* 1994; Premaratna *et al*, 2001; Kochar *et al*, 2003). Possible mechanisms causing hepatic dysfunction include congestion of the hepatic sinusoids caused by PRBC or the effects of cytokines, such as TNF-α, which are released in high concentrations in severe falciparum malaria (Premaratna *et al*, 2001). The study of liver ultrastructural pathology in mice reveals that hepatocyte damage is an important finding associated with advanced stages of *P. berghei* malarial infection, which may lead to liver dysfunction in this disease (Rodriguez-Acosta *et al*, 1998). The ultrastructural lesions encountered in the liver in this study are not different from those reported in earlier studies (Macpherson *et al*, 1985, Mishra *et al*, 1992; Wilairatana *et al*, 1996). It is apparent that Kupffer cells develop hyperplasia with some PRBC within them. Pigment deposition and mononuclear cell infiltration have been observed. Quantitation of WBC in this study showed that WBC (predominantly mononuclear cells) in CM patients were significantly higher than in NCM patients.

Jaundice is a well known manifestation of malaria in adults. In Thailand, 19% of falciparum malaria cases have jaundice, which is predominantly due to unconjugated bilirubin (Wilairatana *et al*, 1994). Jaundice in malaria can be due to intravascular hemolysis, associated septicemia, disseminated intravascular coagulation and hepatocellular jaundice (Harris *et al*, 2001). The results of this study revealed that parasite load in the liver of jaundice patients was significantly higher compared to patients without clinical symptoms. There was a correlation between parasite sequestration in the liver and the level of serum bilirubin. Liver enzymes, including aspartate aminotransferase (AST), alanine aminotransferase and alkaline phosphatase (ALP), were also elevated in malarial patients, indicating hepatocellular damage. There was a correlation between the level of AST and parasite sequestration in the liver. These findings suggested an association between PRBC sequestration in the liver and hepatic dysfunction. The parasite load in the liver of patients with acute renal failure is also significantly higher than in patients without acute renal failure. The mechanism involved may be obstruction by sequestrated PRBC in the liver leading to hepatic dysfunction, particularly jaundice. The association between renal failure and jaundice is a recurrent finding in studies on severe malaria (Mukherjee *et al*, 1971; Nacher *et al*, 2001b). One hypothesis could be that Kupffer cells which develop hyperplasia in patients with high parasite burdens

may have intrahepatic cholestasis. Bilirubin in general is toxic to renal tubular cells (Nacher *et al*, 2001b).

Hepatomegaly is reported to be a common finding in acute primary malaria infection, especially in children, and hyperplasia of the reticuloendothelial cells of the liver occur as part of the response to malaria (Sowunmi, 1996). Our study also showed an association between hepatomegaly and parasite load in the liver. There was a significant correlation between the size of a palpable liver and parasite sequestration in the liver.

The normal function of the spleen is to remove abnormal erythrocytes and intraerythrocytic inclusions. It is clear that the spleen plays a critical role in host defence in malaria, but the precise mechanisms involved are still obscure and remained to be clarified. It has been postulated that parasites avoid the spleen, thereby avoiding exposure to antibodies produced by the spleen and non-specific clearance mechanisms localized in this organ. The role of the spleen in protective immunity mediated by splenic macrophages is often an early feature of the immune response (Pongponratn *et al*, 1989). The present study confirms this finding, in which more phagocytes were found in NCM and jaundice patients. Phagocytosis occurred more predominantly in macrophages than in endothelial cells. Both PRBC and uninfected RBC were preferentially phagocytosed. In the absence of a functioning spleen, RBC inclusions, such as dead or dying malaria parasites, remain in the circulation (Chotivanich *et al*, 2002). The results of this study provide the same evidence as previous studies (Pongponratn *et al*, 1987) that the mechanisms underlying splenic host defence in malaria include both immunological and non-immunological interaction with PRBC. Splenic trapping of PRBC is an important defence mechanism and phagocytosis of both PRBC and RBC probably accounts for anemia and jaundice in these patients. We found numerous evidence of splenic pitting. All parasite stages, including schizonts, were involved in this phenomenon which suggests that this mechanism is the main route of removing circulating parasites. Previous studies comparing the 2 widely used treatments for severe malaria, quinine and artesunate (Chotivanich *et al*, 2000) report that artesunate induced splenic pitting. Our study found pitting with both of quinine and artesunate, but these could not be compared because we did not have data on the number of PRBC showing pitting. To answer this further studies are needed.

Our study is in contrast to the results of other studies (Nacher *et al*, 2001a) suggesting that splenomegaly is associated with the pathogenesis of cerebral malaria. A distinction between the pathogenic and beneficial functions of the spleen is not clear (Weiss, 1989). Perhaps the timing of the opening of the blood-spleen barrier is of importance, with premature opening selecting cytoadhesive clones before the immune response is fully operational (Nacher *et al*, 2001a). There is evidence that the cytoadherence of PRBC to sinusoidal endothelial lining cells occurs in the spleens of patients. This mechanism leads to obstruction of the splenic cords (Pongponratn *et al*, 1987).

Few spleen samples in patients with splenomegaly were examined in this study because of the low prevalence of splenomegaly in Thailand and Vietnam. All our patients were adults. In areas of high transmission, the prevalence of splenomegaly is much higher, especially in children, as they acquire specific immunity. Trapping and congestion in splenic sinusoids, destruction of both parasitized and non-parasitized RBC are presumably responsible for splenomegaly (Pongponratn *et al*, 1987). Our findings showed no relationship between PRBC sequestration in the spleen and splenomegaly.

In acute malaria, the liver and the spleen are congested. There is phagocytosis of infected cells by macrophages and deposition of malaria pigment is predominant (Sowunmi, 1996). Comparative quantitation of parasite sequestration between the liver and spleen showed that the number of RBC and PRBC including K^+PRBC, K^-PRBC and schizonts, were significantly higher in the spleen than in the liver of these patients. This implies that congestion of blood and trapping of PRBC is more commonly seen in splenic sinusoids than in hepatic sinusoids. A significant higher number of phagocytes were observed in the spleen than in the liver. This data provides the same conclusion as previous studies sug-

gesting the spleen is the major site of malaria parasite clearance (Lee *et al*, 1989; Chotivanich *et al*, 2002).

There are several potential problems with the interpretation of ultrastructural studies. The foremost of these are sampling error, sample size and tissue preservation. Tissue ultrastructure is exquisitely sensitive to post mortem degradation. Even with the rapid post mortem times the pathologists was able to achieve in the majority of cases in this series, some patients had poor tissue preservation. However it was found that the presence of parasites in tissue sections was consistent and recognizable. Sampling error is implicit in examining a small fragment of tissue from a large organ. However, separation into multiple grids was an attempt to address this. Small sample size seems to be the main problem of the present study, limiting reliable statistical analysis. Despite the small size, we attempted to demonstrate a relationship between parasite sequestration in different organs with different clinical groups. The significance of these observations needs further study.

In conclusion, the result of the present study suggests an association between PRBC sequestration in the liver and hepatic dysfunction. We have shown that NCM and jaundiced patients had more phagocytes in the spleen, which provides evidence that the spleen is associated with host defence mechanisms against malaria. There was evidence that cytoadherence of PRBC to sinusoidal endothelial cells occurs in the liver and spleen of patients. This leads to obstruction of hepatic and splenic cords. Most phagocytes observed in both organs were MNC. Uninfected RBC, PRBC and malarial pigment were seen phagocytosed in the cytoplasm of these cells. Numerous evidence of splenic pitting was seen, which suggestes that this mechanism is the main route of removing circulating parasites. All parasite stages, including K^-PRBC, K^+PRBC and schizonts, were involved in this phenomenon. Comparative quantitation of parasite sequestration between the liver and the spleen showed that the number of RBC and PRBC including K^+PRBC, K^-PRBC and schizonts, were significantly higher in the spleen than the liver of these patients. This implies that congestion of blood and PRBC trapping are more commonly seen in splenic sinusoids than in hepatic sinusoids. Significantly higher numbers of phagocytes were observed in the spleen than in the liver. This data provides evidence that the spleen is the major site of malaria parasite clearance.

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