ABSENCE OF KNOBS ON PARASITIZED RED BLOOD CELLS IN A SPLENECTOMIZED PATIENT IN FATAL FALCIPARUM MALARIA

Emsri Pongponratn[1], ParnpenViriyavejakul[1], Polrat Wilairatana[2], David Ferguson[3], Urai Chaisri[1], Gareth Turner[4], and Sornchai Looareesuwan[2]

[1]Department of Tropical Pathology; [2]The Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [3]Departments of Cellular Pathology and [4]Cellular Science, The John Radcliffe Hospital, University of Oxford, Oxford, UK

Abstract. We present a case report of fatal falciparum malaria of a splenectomized adult Thai patient. The patient developed high peripheral parasitemia and showed signs of severe malaria with multiorgans involvement. Ultrastructure of *Plasmodium falciparum* -infected red blood cells in a fatal splenectomized patient and pathological features are reported for the first time with special emphasis on the role of the spleen as a modulating cytoadherence phenotype of parasitized red blood cells (PRBC). In this patient, adherence of the PRBC to the vascular endothelium of brain, kidney and lung including blood circulating cells, was noted, despite the absence of knob on the surface of the PRBC.

INTRODUCTION

The spleen plays a central role in the immune response to malaria. It is endowed with the ability to trap and remove damaged and effete red blood cells from the circulation (Schnitzer *et al*, 1972; Pongponratn *et al*, 1989). The spleen is therefore believed to be important in host defence in malaria (Wyler *et al*, 1979; Thu *et al*, 1997). To emphasize the importance of the spleen in immunity to malaria, experimental infection is easier in splenectomized animals (Playfair, 1982).

Of the human malaria parasites, *Plasmodium falciparum* is the only species which sequesters in deep vascular beds. The process of sequestration is characterized by the disappearance of PRBC with the mature forms of the parasite, from peripheral blood and their selective accumulation in internal organs due to adhesion to vascular endothelial cells. Sequestration is thought to favor parasite development in a relatively low-oxygen environment, and allows escape from immune surveillance in the spleen (David *et al*, 1983). Sequestration of parasitized red blood cells (PRBC) in specific organs has been proposed as an important pathophysiological factor in clinical patterns of severe malaria, such as the relationship between cerebral sequestration in brain microvessels and the incidence of cerebral malaria (Riganti *et al*, 1990; Pongponratn *et al*, 1991).

The spleen is thought to modulate the surface expression of parasite antigens involved in cytoadherence and sequestration. Some experimental evidence has shown that PRBC from splenectomized animals did not show knob protein expression and did not sequester (David *et al*, 1983). In another model, two splenectomized Bolivian squirrel monkeys showed marked vascular sequestration and associated pathological lesions similar to spleen intact animals (Whitely *et al*, 1987). Kawai *et al* (1993a) developed Japanese monkeys as a model for severe human malaria with cerebral involvement. They showed that, a splenectomized monkey developed a parasitemia of 20% with clinical signs of severe malaria. At necropsy, it showed a high percentage of PRBC sequestration (> 71%) in microvessels. The sequestered PRBC blocked brain capillaries. Electron microscopic study revealed knobs on the PRBC surfaces that formed focal junctions with capillary endothelial cells. The first report of the *in vitro* ultrastructure of *P. falciparum* PRBC from a splenectomized patient (Ho *et al*, 1992) revealed that all PRBC containing mature trophozoites and schizonts possessed typical knobs on their surfaces. PRBC were found to cytoadhere to C32 melanoma cells.

There have been few case reports of *P. falciparum* infection in asplenic human patients (Garnham, 1970; Maharaj *et al*, 1982; Israeli *et al*, 1987; Petersen *et al*, 1992; Looareesuwan *et*

Correspondence: Professor Sornchai Looareesuwan, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Rajthewi, Bangkok 10400, Thailand.
Tel: (662) 245 8251; Fax: (662) 245 7288
E-mail: tmslr@mahidol.ac.th

al, 1993; Thu *et al*, 1997). These reports showed different individual parasitemia and varied clinical symptoms. There remain several unanswered questions regarding the protective role of the spleen in immunity to malaria, and the effect of splenectomy on the clinical picture of disease. Our study is the first report of a case of fatal falciparum malaria in a splenectomized patient, where autopsy allowed histopathological and ultrastructural examination of tissues to determine the effect of splenectomy on knob expression, sequestration and pathology *in vivo*.

MATERIALS AND METHODS

Case history

This is a case of a 61-year old Thai male who lived for one year in Kanchanaburi Province, Thailand, an area endemic for malaria. His past medical history revealed that he had hypertension for 5 years and diabetes mellitus for 1 year. He was splenectomized following a car accident 10 years ago. No previous exposure to malaria was recorded.

He was first admitted to a private hospital in Kanchanaburi Province because of fever, weakness, drowsiness and later became unconcious with right hand shaking. Computerized tomography scan of the brain was unremarkable. The patient was comatose and needed endotrachial intubation.

A blood smear for malarial parasites was performed and revealed that 73.5% of red blood cells were infected with *Plasmodium falciparum*. A loading dose of intravenous quinine dihydrochloride (20 mg/kg) was given which was followed by intravenous artesunate 60 mg three times daily. Four days after antimalarial treatment, the patient developed tachypnea. Fine crepitation was detected and pulmonary edema was diagnosed. Lasix 40 mg was given. The patient was then referred to the Hospital for Tropical Diseases on the sixth day of illness with stuporose conciousness and extreme weakness. He had no papilledema and no stiffness of the neck.

Treatment given in the Hospital for Tropical Diseases was artesunate 120 mg followed by 60 mg artesunate every 12 hours. On the 9th day of illness, the patient became alert. He could take food by himself. On the 11st day, however his condition deteriorated. He developed oliguria and tachypnoea and became unconcious. Ventivatory support and hemodialysis were instituted with no improvement. He died on the 13rd day of admission. The last parasitemia was 1,195,335 Pf/μl (30.7%). Routine autopsy for histopathological examination was performed 14 hours after death.

Preparation for light and transmission electron microscopy

The tissues were cut and fixed in 10% neutral buffer formalin, dehydrated in graded ethanols, clearing, infiltration in chloroform and embedded in paraffin. Sections were cut and stained with the routine Haematoxylin and Eosin stain before examination by light microscopy.

For transmission electron microscopy, small pieces of brain, heart, lung, kidney and liver were taken from the patient during routine autopsy for histopathological examination. The tissues were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, post fixed with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, dehydrated in graded ethanols, infiltrated with propylene oxide, and embedded in plastic embedding media (Spurr). Thin sections were cut with glass knives and/or diamond knives on the ultramicrotome. Copper grids (200 mesh squares) were used to collect the thin sections which were stained with lead citrate and uranyl acetate prior to examination by transmission electron microscopy (using either JEM-1000 EX II or a Hitachi-H-7000 models).

Analysis of the sections

All tissues were examined with light and electron microscopy. All vessels in the specimens were observed. The total number of red blood cells (RBC) and the proportion containing parasites were counted. Frequency of PRBC in different organs was quantified and expressed as a percentage. Special ultrastructural studies were performed, particularly to determine whether knobs were present on the surfaces of the PRBC.

RESULTS

Clinical findings of the patient are given in Table 1.

Ultrastructural findings

Since autopsy was performed 14 hours after death, the tissue ultrastructure was highly susceptible to degenerative post mortem changes. Changes

Table 1
Clinical findings.

Day of illness	Parasitemia (%)	Hematocrit (%)	WBC x10^9/l	Platelets x10^9/l	Blood urea nitrogen (mg/dl)	Creatinine (mg/dl)	Blood glucose (mg/dl)	Total bilirubin (mg/dl)
1	NA	NA	24	228	16.4	1.5	198	NA
2	NA	NA	14.6	84	NA	NA	NA	NA
3	73.5(Rf)	NA	NA	NA	50.6	2.9	NA	NA
4	48.0(Rf)	NA	NA	NA	51.4	2.5	NA	NA
5	47.0(Rf)	NA	NA	NA	NA	NA	NA	NA
6	29.0(Rf)	NA	NA	NA	42.7	2.45	159	3.44
7	35.8(Rf)	40	20.5	94	38.5	2.85	180	3.2
8	32.6(Rf)	NA	NA	NA	NA	NA	NA	NA
9	35.0(Rf)	39	23.5	200	59.6	3.55	110	NA
10	38.7(Rf)	NA	NA	NA	63.5	3.1	172	2.95
11	33.5(Rf)	33	25	322	74.9	3.3	171	2.95
12	33.1(Rf)	31	24.2	371	54.5	3.52	205	NA
13	30.7(Rf)	32	27.97	379	59.5	4.9	167	6.08

NA = not available, Rf = ring form

commonly seen were the loss of cell membrane and nuclear membrane, and the development of cytoplasmic vacuoles. However despite these changes there were a number of obvious pathological processes seen in this study. Foremost among these was the presence of *P. falciparum*-parasitized red blood cells (PRBC).

It was consistently found that PRBCs revealed no knobs on the surfaces (Fig 1) in every organ examined. Some PRBC possessed irregular membrane outlines, but the majority were regular. No intact parasite was seen in the PRBC. They were recognizable as PRBC only because of the presence of degenerate parasites after antimalarial treatment. The affected parasites showed apparent clumping of cytoplasm similar to that previously described (Fig 1) (Ono *et al*, 1991; Kawai *et al*, 1993b). Frequently, PRBC were recognized only by the presence of cytoplasmic tubulo-vesicular structure (Elford *et al*, 1995) assuming Maurer's clefts in the cytoplasm of PRBC (Fig 2) (Aikawa and Seed, 1980).

In the congested microvasculature, PRBC were seen in intimate contact with endothelial cells. PRBC were also found attached to the aggregation of RBC that appeared in the larger blood vessels (Fig 3). In some venules, PRBC appeared to be marginated along the endothelial cells as well as intermixed with circulating cells. They were in contact with RBC (Fig 3) and other PRBC (Fig 2).

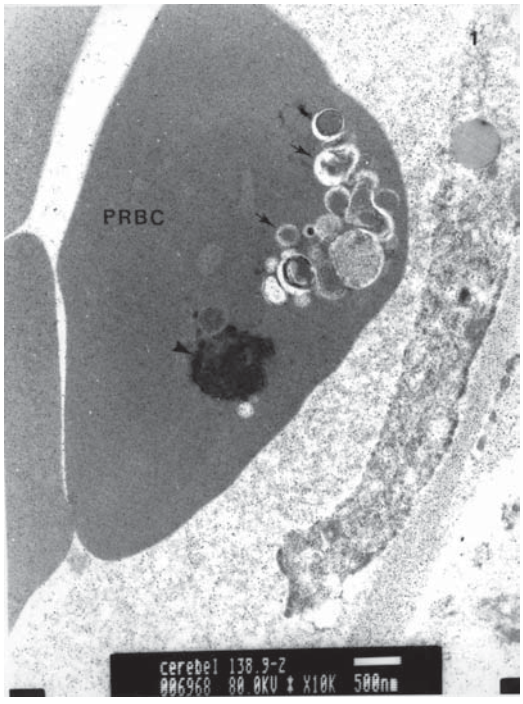

Fig 1–Electron micrograph of a *Plasmodium falciparum*-parasitized red blood cell (PRBC) in a cerebral venule of a splenectomized patient after treatment with artesunate. The PRBC reveals no knob on the surface. A degenerated parasite (arrowhead) is seen in the PRBC cytoplasm. Many cytoplasmic cleft formations in the PRBC are apparent (arrows).

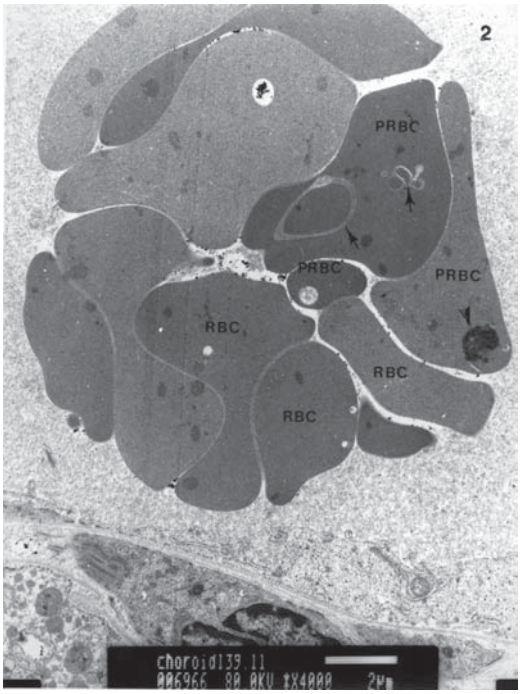

Fig 2–Electron micrograph showing red blood cells (RBC) aggregation in a small cerebral vessel. Parasitized red blood cells (PRBC) are recognizable only with an electron dense mass (arrowhead) and/or cytoplasmic cleft (arrows). The PRBC show close associations to the other PRBC and RBC.

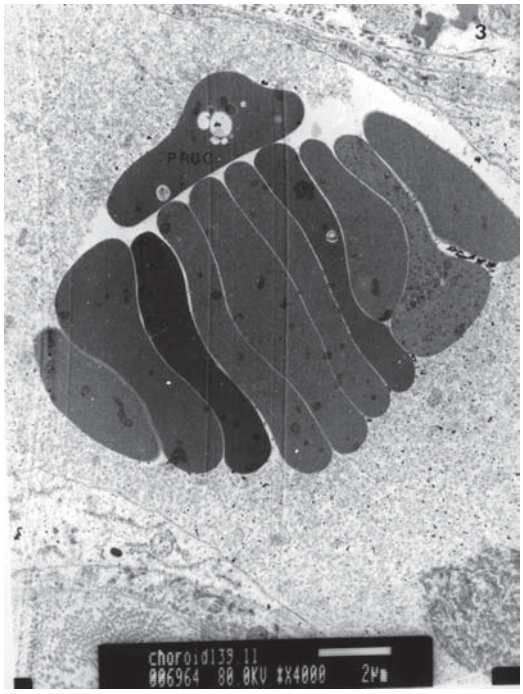

Fig 3–A larger cerebral vessel shows rouleaux formation of red blood cells in the lumen. A parasitized red blood cell (PRBC) does not participate in the formation but adheres to the normal red blood cells.

An interesting feature was the intravascular presence of numerous mononuclear cells in the cerebellum but not in the cerebrum. The mononuclear cells intermixed with RBC were found congested in the cerebellar vessels. However it was not a consistent finding in other parts of the brain. A good number of acute and chronic inflammatory cells were observed in the glomeruli and interstitial tissue of the kidney, in alveoli of the lung and in the choroid plexus (Fig 4). PRBC were always found attached to the mononuclear cells (Fig 4).

The frequency of PRBC in the studied organs was quite high (average, 30.14%; range, 20-50%) (Table 2). In the capillaries, PRBC and RBC were congested and filled the lumen, but in the larger vessels they did not appear to be tightly packed. In the liver, PRBC (Fig 5) and occasionally malarial pigment were seen phagocytosed in the Kupffer cells.

The frequency of PRBC and pathological findings are given in Table 2.

DISCUSSION

The spleen plays a central role in the immune response to malaria. As well as representing the major organ of host defence to intravascular pathogens, it plays a complex role in modulating the separate arms of the immune system, including lymphocyte recirculation and activation by antigen presenting cells of the reticulo-endothelial system. It traps and removes damaged and effete RBC from the circulation (Schnitzer *et al*, 1972; Pongponratn *et al*, 1989). This process is accelerated during malaria infection due to the changes in PRBC shape, deformability and surface structure consequent upon parasitization (Wyler *et al*, 1979). In our study, phagocytosed *P. falciparum* -PRBC (Fig 5) as well as malarial pigment were found in Kupffer cells.

PRBC accumulate in major organs due to adhesion to vascular endothelial cells. Experimental data on this adhesion process shows it to be

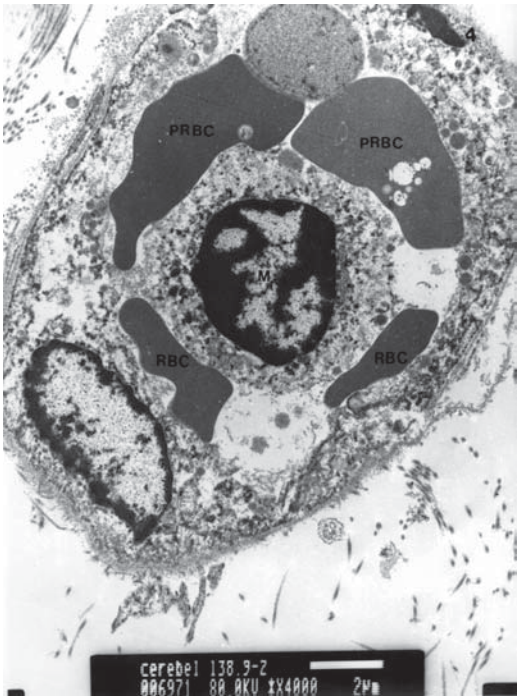

Fig 4–A cerebral microvessel is congested with RBC, PRBC and a mononuclear cell (M).

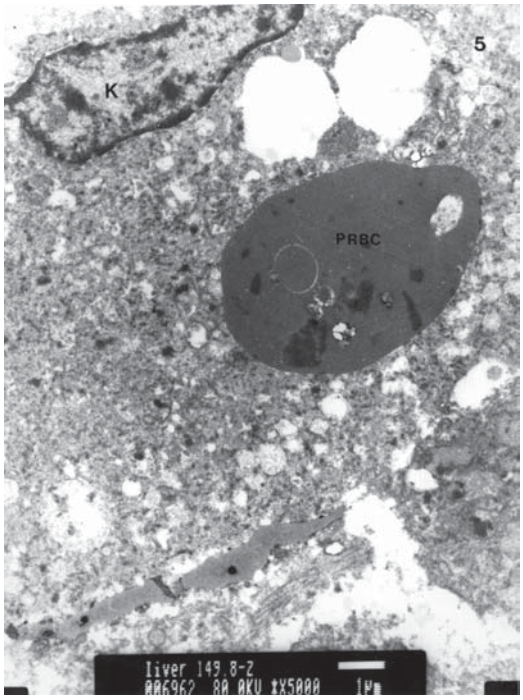

Fig 5–Liver of the splenectomized patient shows a Kupffer cell and phagocytosed PRBC in the cytoplasm.

Table 2
Frequency of PRBC and pathological findings in different organs.

Organ	% PRBC	Pathological findings
Cerebrum	30.27	Congestion.
Cerebellum	30.34	Congestion, moderate mononuclear cells intravascularly.
Kidney	28.74	Acute tubular necrosis, cystic formations, moderate mononuclear cell infiltration.
Lung	50.00	Marked congestion, marked mononuclear cell infiltration.
Heart	20.40	Minute petechial hemorrhages.
Liver	21.12	Centrilobular hemorrhagic necrosis, moderate fatty change, reactive Kupffer cells, minute small cystic formations, moderate chronic inflammation at portal triads.

a specific receptor-mediated event, between parasite encoded adhesins on the PRBC surface, and a range of host endothelial cell adhesion molecules (reviewed by Berendt *et al*, 1994). The parasite proteins involved include PfEMP-1 which is localized under the PRBC membrane at electron dense 'knob' areas (Howard, 1988). Sequestration is thought to favor parasite development in a relatively low-oxygen environment, and allow escape from immune surveillance in the spleen (David *et al*, 1983). Sequestration of PRBC in specific organs has been proposed as an important pathophysiological factor in clinical patterns of severe malaria, such as the relationship between cerebral sequestration in brain microvessels and the incidence of cerebral malaria (Riganti *et al*, 1990; Pongponratn *et al*, 1991).

In this study, ultrastructural findings of *Plasmodium falciparum*-PRBC in our splenectomized

patient failed to show knobs on their surfaces. The spleen is thought to modulate the surface expression of parasite antigens involved in cytoadherence and sequestration. Some experimental evidence has shown that PRBC from splenectomized animals did not show knob protein expression and did not sequester (David *et al*, 1983). However we found a number of PRBC in the vascular beds of the brain, lung, heart and kidney in similar number to those found in peripheral blood parasitemia (Table 2). In one animal model (Whitely *et al*, 1987) splenectomy did not seem to alter the severity of the histologic lesions. In humans, there are parasite strains which lack membrane knob proteins but still show cytoadherence *in vitro* (Udomsangpetch *et al*, 1989). The knob-negative PRBC in our study were seen to be sequestered in the blood vessels by attachment to the vascular endothelium, to the other PRBC and RBC (Figs 2, 3), and to the mononuclear cells (Fig 4). A common feature of all the interactions seen in our study was that the contact points between PRBC and the other cells were simply via apposition of their cell membranes. It is however unclear whether this was a real cytoadherence structure or just simply morphological approximation due to congestion. Several factors may underline this. Further investigation of adhesin localization is needed.

There has never been reported any cases of splenectomized patients with fatal falciparum malaria, and there are only a few case reports of *P. falciparum* infection in asplenic human patients (Garnham, 1970; Maharaj *et al*, 1982; Israeli *et al*, 1987; Ho *et al*, 1992; Petersen *et al*, 1992; Looareesuwan *et al*, 1993; Thu *et al*, 1997). Some of these patients shared a high circulating parasitemia, along with the absence of severe clinical symptoms thought to relate to sequestration. The peripheral blood revealed mature trophozoite and schizont forms, which would normally be sequestered, in addition to younger ring forms (Israeli *et al*, 1987; Looareesuwan *et al*, 1993). One case report did show severe clinical manifestations (Maharaj *et al*, 1982). These findings suggest that the effect of splenectomy in human falciparum malaria is to reduce sequestration with a resultant increase in peripheral parasitemia. However the clinical effects of these changes are varied, perhaps because other pathophysiological mechanisms which occur in severe malaria disease, such as stimulation of host cytokine release, are not sequestration-dependant and may vary with the immune status of the host (Looareesuwan *et al*, 1993).

Artemisinin derivatives have been shown to stop parasite development and inhibit cytoadherence *in vitro* (Udomsangpetch *et al*, 1996). The *in vivo* study by Maeno *et al* (1993) also showed that in Rhesus monkeys experimentally infected with *P. coatneyi*, artesunate interfered with PRBC sequestration and led to morphologically significant changes in the parasites. Disappearance of knobs on the PRBC was noted and the surface malarial protein antigen on the PRBC was not detected. In our splenectomized patient, parasites could still be detected despite 10 days artesunate treatment (Table 2). The level of parasitemia did not change. They showed high peripheral parasitemia of ring forms (average 39%, range 29-73%), similar in number to those PRBC found in the internal organs. These sequestered PRBC contained only dead parasites after artesunate treatment and showed sometimes only Maurer's cleft in the cytoplasm. The PRBC that revealed no knobs on their surfaces could represent failure of the knobs to form, the effect of splenectomy or an artesunate effect. Adhesion of PRBC to endothelium and the other cells found in this study indicates that mechanisms other than knob proteins may play an important role. In this study the finding of a good number of mononuclear cells in the cerebellum, lung and kidney may mean that these cells are involved in the pathogenesis of the symptoms (Patanaik *et al*, 1994).This case developed severe clinical manifestations with multiorgan involvement including cerebral malaria, pulmonary edema and acute renal failure. This report concludes that some event was taking place in this rare case of fatal splenectomized falciparum malaria, and underscores the need to extend the details beyond what is logically interpretable from the electron micrographs. It is important (and sometimes difficult) to be able to distinguish what is real from what is artefact. It is hoped that this report will add information on individual variation of splenectomy in human fatal falciparum malaria.

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