CASE REPORT

PLASMODIUM MALARIAE-INFECTED ERYTHROCYTES IN THE PERIPHERAL BLOOD, LIVER, STOMACH AND DUODENUM: AN ULTRASTRUCTURAL STUDY

Emsri Pongponratn[1], Oranan Prommano[1,3], Urai Chaisri[1], Parnpen Viriyavejakul[1] and Polrat Wilairatana[2,4]

[1]Department of Tropical Pathology, [2]Department of Clinical Tropical Medicine, [4]WHO Collaborating Center for Severe Malaria, Faculty of Tropical Medicine, Mahidol University, Bangkok; [3]Faculty of Medical Technology, Rungsit University, Pathum Thani, Thailand

Abstract. We examined the ultrastructure of *Plasmodium malariae*-infected erythrocytes in peripheral blood and tissue biopsies of the liver, stomach, and duodenum from three patients infected with *P. malariae*. Ultrastructural features of *P. malariae*-infected erythrocytes in peripheral blood appear similar to those described previously. The surface membranes of *P. malariae*-infected erythrocytes had numerous knobs, as seen in *P. falciparum*-infected erythrocytes. There was no evidence of *P. malariae*-infected erythrocytes in the microvessels of the organs. This finding suggests the presence of knobs on *P. malariae*-infected erythrocytes is not associated with the attachment of *P. malariae*-infected erythrocytes to vascular endothelium and may be the reason for the mild symptoms of malariae malaria. The failure to find *P. malariae*-infected erythrocytes in the tissue biopsies using electron microscopy may be due to low parasitemia. More cases with higher parasitemia need to be studied to confirm these findings.

Keywords: *Plasmodium malariae*, quartan malaria, knob, ultrastructure

INTRODUCTION

Malaria is a public health problem in Thailand and in many developing countries, especially in the tropics. Human malaria can be caused by any of five *Plasmodium* species: *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi.* Falciparum malaria is the most severe form of human malaria, causing death more often than the other species. Sequestration of parasitized erythrocytes in the microvasculature of vital organs has been associated with the virulence of *P. falciparum.* (Macpherson *et al*, 1985; Pongponratn *et al*, 1991, 2003).

P. malariae is the third most common type of malaria and is usually found in a low prevalence in Southeast Asia (Kawamoto *et al*, 1999). *P. malariae* malaria comprised <1% of malaria cases in Thailand in

Correspondence: Polrat Wilairatana, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Ratchathiwei, Bangkok 10400, Thailand.
E-mail: tmpwl@mahidol.ac.th

2010 (Ministry of Public Health Thailand, 2010, unpublished data). *P. malariae* generally produces mild disease, but the initial paroxysms can be moderate to severe. It is known for causing chronic infections and is a cause of nephropathy in endemic areas (Hendrickse and Adeniyi, 1979). Malariae malaria is sometimes called "quartan malaria" because it can cause fevers every fourth day. The 72-hour periodicity between fever spikes with *P. malariae* is due to its slower growth and maturation during blood-stage schizogony. *P. malariae* infections are characterized by low parasitemia since they usually invade only aging erythrocytes (McKenzie *et al*, 2001).

Ultrastructural studies of falciparum malarial parasites have been conducted by a number of investigators (Luse and Miller, 1971; Miller, 1972; Aikawa and Seed, 1980; Macpherson *et al*, 1985; Bannister *et al*, 2000). Ultrastructural studies of *P. malariae* malaria infection in humans are uncommon (Smith and Theakston, 1970; Matsumoto *et al*, 1986). The first ultrastructural study of human erythrocytes infected with *P. malariae* was published in 1970 (Smith and Theakston, 1970); they described the surface of *P. malariae*-infected erythrocytes as being covered with numerous knobs and having occasional cytoplasmic clefts, morphologically similar to Maurer's clefts. Similar observations were reported by Atkinson *et al* (1987).

Microvasculature sequestration of *P. falciparum*-infected erythrocytes is thought to play a central role in the pathogenesis of severe falciparum malaria (Raventos-Suarez *et al*, 1985). Although *P. malariae*-infected erythrocytes have numerous knobs, as seen with *P. falciparum*-infected erythrocytes, there have been no reports of cytoadherence or sequestration in

patients-infected with *P. malariae*.

There is no published report of the ultrastructure of *P. malariae*-infected erythrocytes in human tissues. We conducted ultrastructural examination of tissue previously obtained from patients infected with *P. malariae,* evaluating th*e* peripheral blood, liver, duodenum and stomach.

MATERIALS AND METHODS

Patients

Three patients infected with *P. malariae* admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Thailand were retrospective studied. Clinical, biochemical and hematological details of the patients are shown in Table 1. All the patients had a history of fever and headache. The liver was palpable in 2 of 3 patients. These two patients had abnormal liver function test results corresponding to morphological changes of the hepatocytes. The spleen was enlarged in the third patient. One patient had a previous history of malaria infection two years prior to admission. The patients were treated with a complete course of oral chloroquine; two of the patients were also treated with artesunate and mefloquine. All the patients were hospitalized until the parasitemia was resolved.

P. malariae parasites were detected in the peripheral blood by an experienced microscopist. All the patients had 0.1-0.2% parasitemia. For ultrastructural studies of the *P. malariae*-infected blood, we collected the blood in heparin by venipuncture soon after infection was diagnosed. Tissue biopsies of the liver, stomach, and duodenum for histopathological examination were used for the ultrastructural studies.

The protocol for tissue collection was

Table 1
Patient demographic data.

	Patient 1[a]	Patient 2 (range)	Patient 3 (range)	Normal range
Sex	Female	Female	Male	-
Age (years)	23	18	18	-
Parasite count (/mm^3)	1,320	1,440	1,560	-
RBC (x10^{12}/l)	3.45	4.89 (4.67-5.2)	4.36 (3.92-4.86)	4.0-6.0
WBC (x10^9/l)	3.7	7.8 (6.2-9.2)	5.96 (3.1-7.5)	5.0-10.0
Hb (g/dl)	8.7	10.1 (9.5-10.9)	13.8 (12.7-15.2)	12-18
PCV (%)	27	32.5 (30-36)	39.8 (36-46)	37-54
MCV (fl)	79	65.75 (64-67)	91.6 (87-95)	82-96
MCHC (g/l)	32	31.25 (30-32)	34.8 (33-36)	32-36
Platelet count (x10^9/l)	168	271.75 (186-371)	224 (116-308)	200-500
Blood glucose (mg/dl)	79	93.5 (80-117)	81.2 (78-86)	75-110
BUN (mg/dl)	13.0	9.1 (7.5-12.5)	12.18 (9.2-17)	5-19
Creatinine (mg/dl)	1.1	0.66 (0.55-0.7)	1.00 (0.93-1.17)	0.6-1.4
Total bilirubin (mg/dl)	1.2	0.71 (0.25-1.30)	0.57 (0.37-0.84)	0.1-1.2
Direct bilirubin (mg/dl)	0.25	0.17 (0.04-0.25)	0.14 (0.12-0.18)	0.30-0.52
Total protein (g/dl)	ND	ND	7.19 (6.9-7.5)	6.3-8.0
Albumin (g/dl)	3.9	4.24 (4.0-4.4)	4.02 (3.8-4.3)	3.5-5.0
Alkaline phosphatase (U/l)	73	126.66 (124-153)	106.8 (89-129)	9-35
AST (U/l)	20	83 (32-116)	78.6 (58-101)	7-40
ALT (U/l)	18	128.5 (63-186)	159.8 (118-201)	7-40
Cholesterol (mg/dl)	88	111.75 (82-174)	120.2 (78-138)	150-250
Na$^+$ (mmol/l)	139	139.25 (137-141)	140.6 (140-141)	135-148
K$^+$ (mmol/l)	2.9	4.33 (3.8-4.7)	3.94 (3.7-4.1)	3.5-5.3
Cl$^-$ (mmol/l)	104	106.25 (104-108)	103.6 (102-105)	98-110
HCO$_3^-$ (mmol/l)	24	22.25 (21-23)	27.75 (27-28)	22-33

[a]The patient was examined for hematological and biochemical data only on day 0 of admission.

reviewed and the study was approved by the Ethics Committee, Faculty of Tropical Medicine, Mahidol University (No. TM-IRB 024/2002).

Histopathologic studies

The tissue specimens were immersion-fixed in 10% buffered neutral formalin, pH 7.4, dehydrated in graded ethanol, and embedded in paraffin. Three to five micrometer sections were cut and stained with hematoxylin and eosin and Giemsa for light microscopy.

Ultrastructural studies

One portion of heparinized blood sample was fixed with 2.5% glutaraldehyde in 0.1 M phosphate sucrose buffer, pH 7.4 for at least 1 hour. The fixed cells were washed three times with 0.1 M phosphate buffer, pH 7.4, post fixed with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, at 4°C for 1 hour, dehydrated in graded ethanol, 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 examination with a transmission electron microscope (Hitachi H-7000 model, Tokyo, Japan).

The tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for at least 2 hours. The fixed tissues were subsequently washed three times with 0.1 M phosphate buffer, pH 7.4 and processed as described earlier.

RESULTS

Diagnosis of *Plasmodium malariae* infection

The patients were diagnosed with *P. malariae* infection by thick and thin blood films stained with Giemsa stain. All slides contained all stages of malarial parasites, including band forms of trophozoites, which are typical of *P. malariae* parasites. The percent parasitemia was low in all patients.

Laboratory findings

The averages for the biochemical and hematological values are shown in Table 1. The serum transaminases and alkaline phosphatase in patients 2 and 3 were markedly increased. Other biochemical findings were not remarkable. Patients 1 and 2 had anemia without jaundice.

Ultrastructure of *P. malariae*-infected erythrocytes in peripheral blood

Erythrocytes infected with asexual parasites were easily distinguished from normal erythrocytes on electron microscopy. Asexual parasites observed were trophozoites and very young stage parasites. The erythrocytes infected with mature malarial parasites showed numerous membrane protrusions, or "knob" (Fig 1a). Sections of the infected erythro-
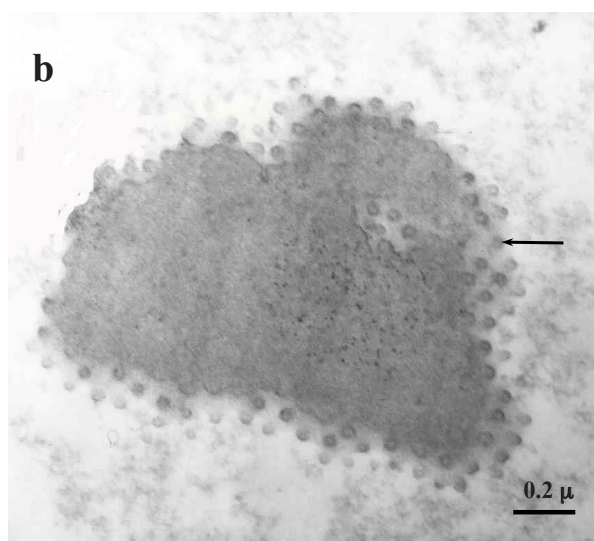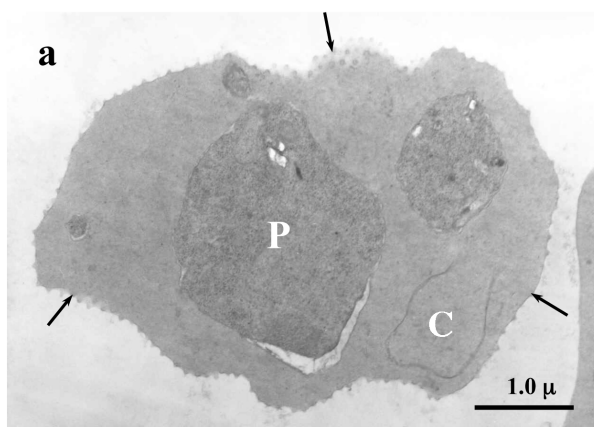

Fig 1–a) Human erythrocyte infected with *P. malariae* (P) showing numerous knobs (←). Also seen is cytoplasmic cleft (C). b) Tangential cut of knobs (←) of *P. malariae*-infected erythrocyte.

cytes demonstrated the knobs were cone shaped (Fig 1a) and composed of electron-dense material that fused gradually into the red cell cytoplasm. On the tangential cuts, the knobs were round and smooth (Fig 1b). These knobs were not seen in the uninfected erythrocytes or erythrocytes infected with young stage parasites.

Other morphological features included the presence of cytoplasmic clefts (Fig 1a) in the infected erythrocytes. The clefts were surrounded by a unit membrane, extended through the cytoplasm of the infected erythrocytes.

P. malariae-infected erythrocytes in tissue biopsies

Light microscopy of the liver revealed mild degeneration of hepatocytes and fatty change. Kupffer cells were reactive. Some Kupffer cells had engulfed malarial pigment within them. The stomach and duodenum had vascular congestion. A good number of eosinophils were seen. Plasma cells and lymphocytes were increased in the lamina propria. Few infected erythrocytes or malarial pigments were observed in liver, stomach and duodenum.

No infected erythrocytes were seen within blood vessels or at hemorrhage sites of the organs under electron microscope.

DISCUSSION

Although, *P. malariae* infection is thought to be an important cause of nephropathy in many tropical countries (Hendrickse and Adeniyi, 1979), our findings did not support this; we found no evidence of abnormal renal function. There are no published reports of the ultrastructure of *P. malariae*-infected erythrocytes in human tissue. Morphological changes of hepatocytes in the patients of our study were observed. These changes were possibly associated with the minor abnormal liver function as seen in our previous report (Tangpukdee *et al*, 2006).

Several ultrastructural morphological changes in infected erythrocytes have been observed among *Plasmodium* spp of primate malaria parasites, such as knobs, caveolae, caveola-vesicle complexes, cytoplasmic clefts, and electron-dense material (Atkinson and Aikawa, 1990). Ultrastructural features of *P. malariae*-infected erythrocytes in peripheral blood of our patients in this study appear similar to those described previously in other human malarial parasites. The surface membrane of *P. malariae*-infected erythrocytes had knobs, as seen in *P. falciparum*-infected erythrocytes and *P. ovale*-infected erythrocytes (Matsumoto *et al*, 1986). Although similar membrane knobs have been demonstrated by other studies (Smith and Theakston, 1970; Atkinson *et al*, 1987). Mackenstedt *et al* (1989) reported the knobs were not seen on the surface of *P. malariae*-infected erythrocytes. The invaginations and microvesicles seen among *P. malariae*-infected erythrocytes corresponded to the morphological alterations induced by *P. vivax*. These morphological alterations were not seen in our study. Our study failed to detect caveolae on the membrane of host erythrocytes, while a study by Atkinson *et al* (1987) found caveolae similar to those described in primate parasites, *P. coatneyi* and *P. knowlesi*. They suggested the caveolae might correspond to Ziemann's stipping (Atkinson *et al*, 1987).

The knobs on the surface of *P. falciparum*-infected erythrocytes are believed to play a role in the sequestration of *P. falciparum*-infected erythrocytes since they were points of contact between *P. falciparum*-infected erythrocytes and vascular endothelial cells leading to microvascular obstruction (Raventos-Suarez *et al*, 1985). Parasite species which express knobs, usually exhibit the highest level of sequestration and are associated with disease severity in severe falciparum malaria. In our study knobs were not as-

sociated with sequestration in *P. malariae* infected patients.

There was no evidence of *P. malariae*-infected erythrocytes in the microvessels of the organs studied. This suggests the presence of knobs on *P. malariae*-infected erythrocytes is not associated with the attachment of *P. malariae*-infected erythrocytes to vascular endothelium and may be the reason for the mild symptoms of malariae malaria. In our electron microscopic study, there were only small pieces of tissue left over from the histopathological examination. The failure to find *P. malariae*-infected erythrocytes in the tissue biopsies using electron microscopy despite examining over 20 grid squares from 2-3 blocks may be due to the low parasitemia in our patients. More cases with higher parasitemia need to be studied to confirm these findings.

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