CONCISE COMMUNICATION

Central Role of the Spleen in Malaria Parasite Clearance

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In acute malaria, red blood cells (RBCs) that have been parasitized, but no longer contain a malaria parasite, are found in the circulation (ring-infected erythrocyte surface antigen [RESA]–RBCs). These are thought to arise by splenic removal of dead or damaged intraerythrocytic parasites and return of the intact RBCs to the circulation. In a study of 5 patients with acute falciparum malaria who had previously undergone splenectomy, it was found that none of these 5 patients had any circulating RESA-RBCs, in contrast to the uniform finding of RESA-RBCs in all patients with acute malaria and intact spleens. Parasite clearance after artesunate treatment was markedly prolonged, although the parasites appeared to be dead and could not be cultured ex vivo. These observations confirm the central role of the spleen in the clearance of parasitized RBCs after antimalarial treatment with an artemisinin derivative. Current criteria for high-grade antimalarial drug resistance that are based on changes in parasitemia are not appropriate for asplenic patients.

The normal function of the spleen is to remove abnormal erythrocytes and intraerythrocytic inclusions. Malaria-infected red blood cells (RBCs) contain an increasingly large and rigid parasite. Starting at ~13–16 h and peaking toward the middle of the asexual life cycle (24 h), the parasitized RBCs adhere to vascular endothelium and thereby avoid splenic removal. The younger ring-stage parasite is small and flexible and does not perturb the membrane configuration of RBCs or express parasite antigens externally. The more mature stages (the mature trophozoites and schizonts), which are larger, change the discoid shape of the infected RBCs and alter the host RBC membrane by the insertion of neoantigens, such as the ring-infected erythrocyte surface antigen (RESA) and *Plasmodium falciparum* erythrocyte membrane protein 1 (Pf EMP 1). The antigenic parasite adhesin Pf EMP 1 is expressed on the exterior of these RBCs. These various changes result in a loss of RBC deformability and an increase in antigenicity. Damage to the malaria parasite, as a result of either drug treatment or host defense mechanisms, leads to parasite clearance. We recently provided evidence that the principal mechanism of parasite clearance, particularly after treatment with artemisinin derivatives, is removal of intraerythrocytic malaria parasites without destruction of the RBCs [1, 2]. In this study, we explore the role of the spleen in this process.

Patients and Methods

Patients. During the course of prospective studies on the treatment of acute falciparum malaria, we studied 5 patients who had previously undergone splenectomy: 2 with severe malaria and 3 with uncomplicated infections. Two of them were admitted to the Hospital for Tropical Diseases, Bangkok, 2 were admitted to the Shoklo Malaria Research Unit, and 1 was admitted to Mae Sot Hospital, both of the latter in Tak Province, northwest Thailand. Severe malaria was defined by World Health Organization criteria [3]. The reasons for earlier splenectomy were blunt trauma (3 cases), gunshot (1 case), and massive splenomegaly (β-thalasssemia; 1 case). The 3 patients with uncomplicated malaria received oral artesunate (12 mg/kg body weight) combined with oral mefloquine (25 mg/kg), and the 2 with severe malaria were treated with intravenous artesunate (120 mg; then 60 mg every 12 h). None had had blood transfusions in the previous 3 months. Each gave fully informed consent to blood sampling.

Blood samples. Thin blood films obtained from *P. falciparum*–infected patients at hospital admission and after antimalarial drug treatment were stained with Giemsa's stain for determination of parasite density and parasite staging, as described elsewhere [4]. Unstained blood films were fixed with absolute methanol, air dried, and kept at −20°C for detection of the RESA. In addition, thin blood films from 2 of the 5 splenectomized patients were prepared

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These studies were part of clinical studies approved by the Ethical and Scientific Review Committee of the Research Committee, Ministry of Public Health, Royal Government of Thailand. Fully informed consent for blood sampling was obtained from the patient or attendant relative.

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Table 1. Patient characteristics at hospital admission.

Patient	Age, years	Sex	Parasites/μL	PCT, days	Hematocrit, %	Serum creatinine level, mg/dL	Diagnosis
1	37	F	233,616	56	31	NA	Uncomplicated malaria
2	12	M	704,616	63	33	NA	Uncomplicated malaria
3[a]	61	M	1,456,960	—	40	2.45	Cerebral malaria
4	36	M	442,112	>5	40	1.2	Severe malaria
5	40	M	111,030	>3	52	1	Uncomplicated malaria

NOTE. NA, not available; PCT, parasite clearance time (the interval between beginning antimalarial treatment and the first negative blood slide).

[a] Patient died.

daily in series after treatment, as described above, for parasite count and detection of RESA cells.

Splenic blood smears. To examine parasitized RBC distribution in the spleen, samples (blood smears from splenic tissue) obtained during a routine autopsy performed on a separate patient with a fatal case of cerebral malaria who had been admitted to the Hospital for Tropical Diseases were examined. The patient had received parenteral artesunate. The autopsy was performed within 3 h after cardiac arrest. Blood smears were prepared from a fresh section of spleen during the autopsy. The blood smears were fixed with methanol and kept at −20°C for detection of RESA.

Determination of RESA. A demarcated area of the methanol-fixed blood smears was labeled and was incubated with mouse monoclonal antibody (1F1) to RESA [5] (a kind gift from Dr. Klaus Berzins, University of Stockholm) and with pooled human hyperimmune serum for 30 min at room temperature in a moist chamber. After washing in PBS, the antibody bound on the erythrocytes was detected by incubating the smears with a rabbit antibody to mouse immunoglobulin and/or with fluorescein isothiocyanate–conjugated human immunoglobulin for 30 min, under the same conditions as described above. The smears were then rinsed gently in PBS and were mounted with 50% glycerol-PBS for examination with the 100× objective of an UV emission light microscope (Olympus Optical Co.).

Parasitemia (parasites/μL) was calculated using the following formula: % hematocrit × 125.6 × number of parasitized cells/1000 RBCs. The numbers of RESA-positive infected RBCs (RESA-PRBCs) and RESA-positive parasite-negative RBCs (RESA-RBCs) were counted per 5000 RBCs at hospital admission and after treatment.

Parasite culture. Blood samples that were still malaria-smear positive, obtained from 2 of the splenectomized patients after antimalarial treatment, were prepared for continuous in vitro culture, using standard methods [6]. During the continuous culture, the thin blood films were prepared for microscopic examination (parasite density and shape) each day, and RESA cells were counted weekly.

Results

Patients. Patient characteristics at admission are presented in table 1. The mean age was 34.6 years (range, 12–61 years). Of the 5 patients, 3 had uncomplicated malaria, 1 had severe malaria with hyperparasitemia but no cerebral involvement, and 1 had cerebral malaria. The geometric mean parasitemia at admission was 356,713 parasites/μL (range, 111,030–1,456,960 parasites/μL), and the mean hematocrit at admission was 40% (range, 31%–52%). Of the 4 survivors, 1 was admitted and stayed in the hospital for 3 days; the others were treated in the outpatient clinic.

The patient who died was admitted with a diagnosis of cerebral malaria and died on the fifth day after admission of pulmonary edema and acute renal failure. The parasitemia at admission was 1,456,960 parasites/μL and before death was 1,398,570 parasites/μL.

Parasite clearance. The blood films of all splenectomized patients suggested the presence of asplenia, with marked anisocytosis and Howell Jolly bodies. The patient with β-thalassemia also had microcytosis and many target cells. Parasite clearance times in the 4 surviving splenectomized patients were prolonged after antimalarial treatment. Malaria parasites were observed in blood films until 60 days after treatment in 2 patients, as shown in figure 1. Following treatment, the parasitemia declined slowly, and morphology of the parasites in the blood films, with pyknotic nuclei and shrunken cytoplasm, suggested that they were dead. These morphological changes were similar to those seen after exposure to 1 μg/L of artesunate in vitro.

RESA cell determination. A mouse monoclonal antibody showing restricted recognition of the ring-stage *P. falciparum*–derived antigen as RESA [5, 8] was used, as described elsewhere

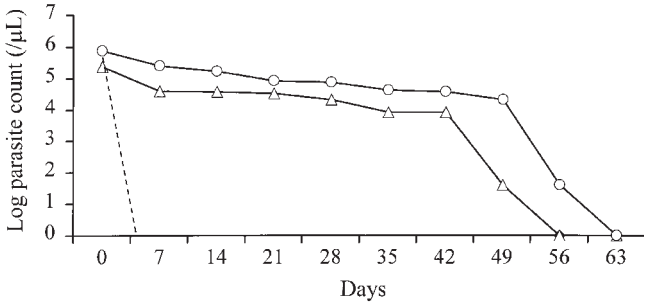

Figure 1. Parasite clearance profile in patients acutely infected with *Plasmodium falciparum* after treatment with artesunate. Shown are data for patients with intact spleens (*broken line* [7]) and for splenectomized patients (*unbroken lines; n* = 2).

[1, 2]. Pooled human hyperimmune serum stained positively, with a pattern similar to the pattern obtained with mouse monoclonal antibody (1F1). The ring-infected RBCs, on thin smears, all showed the characteristic pattern of RESA staining. The geometric mean number of RESA-PRBCs at admission was 157,377 cells/μL (range, 23,361–1,758,400 cells/μL). However, this pattern of staining was not seen in any of the RESA-RBCs in the blood smears of the splenectomized patients (either at admission or in slides taken after treatment).

Splenic findings at autopsy in a separate fatal case. This 22-year-old man was admitted with cerebral malaria. His hematocrit was 43%, and his serum creatinine level was 0.8 mg/dL. The parasite density at admission was 175,000 parasites/μL. The parasites were all in the ring stage, with an estimated mean age of 6 h. Immunostaining of peripheral blood smears at admission showed a fluorescent RESA staining pattern on the ring-infected RBCs (86,413 cells/μL). The RESA staining pattern was also observed on uninfected RBCs (54,008 cells/μL; ratio 62%). The patient died after 36 h of treatment with intravenous artesunate. By this time, the parasite density had declined to 40 parasites/μL, and the density of RESA-RBCs in the peripheral blood had risen to 108,016 RESA-RBCs/μL. The cause of death was cardiorespiratory arrest. In the spleen-imprinted blood smear taken at autopsy, ring-infected RBCs were scanty (3240 ring-infected RBCs/μL), and RESA-staining RBCs were also seen (4860 RESA-RBCs/μL; ratio, 150%).

In vitro culture. Blood samples from 2 splenectomized patients after treatment with artesunate were collected weekly in an attempt to culture the parasites, using standard methods [6]. No growth was achieved. The pyknotic intraerythrocytic parasites persisted for a week in vitro.

Discussion

The spleen plays a pivotal role in policing the circulating RBC population, removing RBCs that are coated with antibody or have reduced deformability and extracting intracytoplasmic particulate material, such as nuclear remnants (Howell Jolly bodies) or oxidized hemoglobin (Heinz bodies). Splenic clearance function is increased in malaria [9, 10]. In the absence of a functioning spleen, RBC inclusions, such as dead or dying malaria parasites, remain in the circulation. The shape of the persistently circulating parasites in the asplenic patients reported in this study was similar to that induced in vitro by exposure to artesunate. The parasite cytoplasm appeared to be shrunken and dense, and the nucleus appeared to be pyknotic.

The parasite-derived antigen Pf 155, or RESA, is inserted in the RBC membrane in the early stage of infection and acts as a "footprint" of parasitization by *P. falciparum*. In earlier studies [1, 2, 11], RESA-RBCs were found in all patients with acute *P. falciparum* infection (45 with uncomplicated and 115 with severe malaria, all with intact spleens), and the number of these cells rose after antimalarial drug treatment, as parasitemia declined.

In this series of 5 patients who had previously undergone splenectomy, there were no RESA-RBCs, and there was no evidence of removal of intraerythrocytic parasites. Indeed, RBCs containing dead parasites continued to circulate for months after treatment in 2 patients who could be followed up. The normal spleen presumably reacts to the drug-affected parasite in the same way it reacts to nuclear remnants or other intraerythrocytic particles and removes them without destroying the RBC. The autopsy findings in a separate patient with an intact spleen who died of cerebral malaria appeared to confirm this. There was a higher proportion of parasites and, in particular, a higher ratio of RESA-RBCs, compared with parasitized cells, than in the peripheral blood. In the absence of the spleen, there is no mechanism to remove the dead parasites and return the intact RBCs to the circulation.

The present study supports our previous investigations, in which the rapid clearance of parasitized RBCs after artesunate treatment was associated with an increase in the number of RESA-RBCs in the circulation [2], and confirms that the spleen is the site of circulating parasite clearance. Presumably, parasite clearance is also prolonged in patients with impaired splenic function but an intact spleen, and this may explain the occasional finding of prolonged parasite clearance after treatment with an artemisinin derivative. The proportion of RESA-RBCs could be used to distinguish hyposplenism from drug resistance. Although the drug-affected parasites have clearly abnormal shapes, distinguishing live from dead parasites does require considerable experience with malaria microscopy. There was no evidence that these parasites were still viable. Thus, the conventional criteria for high-grade antimalarial drug resistance (i.e., failure of parasitemia to fall by 75% in 48 h after the start of treatment) cannot be used in asplenic patients. Patients without a functional spleen who have prolonged parasite clearance presumably do not require additional antimalarial treatment.

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