In Vivo Removal of Malaria Parasites From Red Blood Cells Without Their Destruction in Acute Falciparum Malaria

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During acute falciparum malaria infection, red blood cells (RBC) containing abundant ring-infected erythrocyte surface antigen (Pf 155 or RESA), but no intracellular parasites, are present in the circulation. These RESA-positive parasite negative RBC are not seen in parasite cultures in vitro. This indicates that in acute falciparum malaria there is active removal of intraerythrocytic parasites by a host mechanism in vivo (probably the spleen) without destruction of the parasitized RBC. This may explain the observed disparity between the drop in hematocrit and decrease in parasite count in some hyperparasitemic patients. The fate of these ''once-parasitized'' RBC in vivo is not known.

THE MECHANISMS of parasite clearance in human malaria in vivo are unclear. The spleen is considered the major site of parasite removal[1] and splenic function is increased in acute malaria; the clearance thresholds for both mechanical filtration and Fc receptor-mediated parasitized erythrocyte clearance are lowered.[2,3] Parasite clearance is delayed considerably in splenectomized patients. Despite evidence from animal studies in the 1960s that the spleen could remove intraerythrocytic parasites leaving the host erythrocyte intact, a process known as pitting, parasite clearance in human malaria has been considered generally to result in obligatory destruction of the parasitized RBC.[4] Anemia is an inevitable consequence of malaria infection,[1] but we have observed that the hematocrit in some patients with heavy parasitemias does not decrease as much as would be expected from destruction of the parasitized RBC (unpublished observations, 1992). This is particularly evident with the rapid parasite clearance that follows treatment with artemisinin or one of its derivatives. To establish whether pitting occurs in human malaria, we have used detection of the Pf 155 or Ring Erythrocyte Surface antigen (RESA), which is expressed immediately following merozoite invasion, as a measure of parasitization and correlated this with the presence of intraerythrocytic parasites.

MATERIALS AND METHODS

Preparation of cells. Plasmodium falciparum–infected RBC were obtained from admission blood samples from patients with uncomplicated (n = 22) and severe (n = 26) falciparum malaria admitted to Mae Sot Hospital, Tak Province, western Thailand or the Hospital for Tropical Diseases, Bangkok. The World Health Organization criteria[1] were used to define severe malaria. Patients requiring hospitalization, but without one of these criteria, were considered to have uncomplicated falciparum malaria. These patients had no previous antimalarial treatment and were enrolled in prospective studies of malaria treatment, which will be described in detail elsewhere. Blood samples were also taken for full blood count, routine biochemistry, glucose and lactate measurements. A laboratory strain of *P falciparum*, TM267R, cultured in O^+ RBC (RBC) for greater than 3 years was used as a standard comparator. Blood samples containing ring-stage parasites were washed three times in isotonic saline solution, the buffy coat was removed, and the packed RBCs were resuspended to 50% hematocrit in heat-inactivated fetal calf serum. Thin blood films were made from the RBC suspension, air-dried, and fixed with methanol. In some assays, the thin films were fixed with 1% glutaraldehyde in saline solution, washed, and air-dried. All fixed thin film slides were stored at −20°C until use. Admission samples from patients with *P vivax* malaria infection and also samples from healthy donors were processed and stored as described above and used as negative controls.

In vitro culture. During the continuous in vitro culture, the patients' RBCs were replaced progressively with normal erythrocytes from healthy O^+ donors, and ring-stage parasites in these normal RBCs were maintained in vitro using continuous culture conditions.[5] Ring-infected RBCs from these cultures were then used to prepare samples as described above. The following antibodies were tested: (1) pooled sera from *P falciparum*-immune Thai donors (kindly provided by S. Thaithong, Chulalongkorn University, Bangkok, Thailand); (2) a human monoclonal antibody (MoAb) 3362 recognizing the *P falciparum* RESA antigen (RU 1986)[6]; (3) serum from a *P vivax*–infected Thai patient; and (4) serum from a healthy Thai donor with no previous malaria exposure. All sera were heat inactivated and absorbed twice against group AB^+ RBCs to deplete anti-RBC antibodies that might interfere in the assay.

Antibody staining. The antisera were used at 1:50 dilution. Each antibody (5 μL) was placed on a demarcated area on the thin blood films, incubated in a humidified chamber for 30 minutes at room temperature, and washed twice in isotonic saline solution. Rabbit antihuman immunoglobulin-G (5 μL) conjugated with fluorescein isothiocyanate (FITC) was then added to the previously stained area on the thin films and incubated for 30 minutes under the same conditions as above. After washing, all thin films were mounted with 50% glycerol in saline solution containing 10 ug/mL ethidium bromide to visualize the intraerythrocytic parasite DNA. The stained thin films were then examined with an ultraviolet (UV) light microscope at 1,000× magnification. The FITC-stained ring-infected RBCs and the number of FITC-stained uninfected RBCs (RESA-RBC) were determined and expressed as numbers/1,000 RBCs.

In the patients whose RBCs were used for cell preparation, serum was also tested at a single dilution (1:50) for the presence of antibodies to RESA using the staining procedures as described above.

Statistical analysis. Data were analyzed using Statview 4.1 (Abacus Concepts Inc, Palo Alto, CA, 1994). Normally distributed data were compared by the Student's *t*-test and non-normally distributed parameters were compared using Mann-Whitney U test. Corre-

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lations were assessed by Spearman's method. The χ^2 test with Yates' correction was used for comparison of groups.

RESULTS

Laboratory isolate. Glutaraldehyde-fixed or methanol-fixed thin blood films of ring-infected RBCs from a laboratory parasite strain TM267R were tested with pooled sera of *P falciparum* immune donors, and the human MoAb 33G2 to RESA. Both antibodies reacted with only the membrane of ring-infected RBCs and gave similar intensity and pattern of staining. This indicated that fixation of blood films with either glutaraldehyde or methanol provided equal antibody access to the RESA and that immune sera could substitute for the MoAb in routine staining.

All sera preabsorbed with AB^+ + RBCs showed no reactivity with normal RBCs in the indirect immunofluorescence assay. Serum from a *P vivax*–infected patient and a healthy donor did not stain the ring form-infected RBCs. None of the antibodies stained uninfected RBCs in the TM267R parasite culture.

Acute malaria. Of the 48 patients studied, 26 had severe falciparum malaria (12 cerebral malaria, 3 acute renal failure, 1 severe anemia, 2 jaundice, 8 hyperparasitemia) and the other 22 had uncomplicated malaria. Four patients with severe malaria (15%) died. The geometric mean (range) parasite counts were higher in the severe malaria group; 174,830/μL (3,010 to 1,210,920) compared with the uncomplicated group; 26,540/μL (2,070 to 221,560); $P < .05$. The mean (standard deviation [SD]) hematocrit was similar in the severe and uncomplicated malaria groups; 32 (9)% and 35 (8)%, respectively. The clinical and laboratory values for the patients with severe malaria are shown in Table 1. Thin blood films from malaria patients on admission that contained bright ethidium bromide-stained parasite nucleic acid were then stained with the pooled immune sera or MoAb 33G2 and showed the typical RESA pattern of staining on the infected RBCs. This pattern was also seen on some uninfected RBCs (RESA-RBC) containing no ethidium bromide (Fig 1). The absolute number of RESA-RBC was higher in patients with severe malaria (geometric mean [range] 21,720/μL [5,650 to 166,550] compared with 7,200/μL [2,390 to 39,190] in patients with uncomplicated malaria. The median (range) ratio of parasitized RBCs to RESA-RBCs was 8.63 (0.133 to 50) for severe patients and 2.917 (0.278 to 20) for uncomplicated patients ($P < .05$). There was a significant positive correlation between absolute parasitemia and absolute RESA-RBC numbers ($r^2 = .52$, $P = .0004$), ie, RESA-RBC accounted for 50% of the variation of parasitemia (Fig 2). There were no significant correlations for any other markers of disease severity including spleen size, hematocrit, lactate, or glucose.[1,7] There was no significant difference for duration of previous illness before presentation or for outcome.

Continuous in vitro culture. When all of the patients' RBCs had been replaced by RBCs from a healthy donor in continuous culture, the infected RBCs stained as before with pooled immune sera or human MoAb 33G2, but either no RESA-RBC was detected or, in eight patients' cultures, very low numbers were seen (median, 100 cells/mL, range, 100 to 500). There was no significant difference in the numbers of RESA-RBC between mild and severe patients in vitro.

Anti-RESA antibodies. Sera from *P falciparum*–infected patients were tested for antibodies to RESA by indirect immunofluorescence assay. Thirteen sera showed reactivity with RESA at 1 in 25 dilution. Ten patients had uncomplicated disease and three had severe disease ($P = .011$) There was no significant difference in the number of RESA-RBC or measures of diseases severity between the antibody positive and antibody negative patients, although there was a significantly lower parasitemia in the antibody positive patients ($P = .009$).

Table 1. Summary of Clinical and Laboratory Variables in 26 Patients With Severe Falciparum Malaria (median, range unless indicated)

Temperature	38.8
(°C)	(36.5-40)
Pulse	111
(beats/min)	(88-140)
Systolic blood pressure	100
(mm Hg)	(80-140)
Respiratory rate	35
(breaths/min)	(20-48)
Glasgow coma score	15
	(3-15)
Parasite count/mL	174,830
geometric mean (range)	(3,010-1,210,910)
RESA-RBC count/μL	21,720
geometric mean (range)	(5,650-166,550)
Ratio parasite count to RESA-RBC	8.63
median (range)	(0.2-75)
Lactate (mmol/L)	6.8
mean, (SD)	(5.3)
Blood urea nitrogen	27.5
(mg/dL)	(12-108)
Serum creatinine	1.15
(mg/dL)	(0.6-7.6)
Total bilirubin	2.85
(mg/dL)	(0.6-23.3)
SGOT	49
(U/L)	(18-355)
SGPT	20
(U/L)	(5-91)
White cell count	7.6
(cells $\times 10^9$/L)	(4.7-8.8)
Hemoglobin	9.9
(g/dL)	(5.5-15.2)
Platelet count	93
(cells $\times 10^9$/L)	(20-191)

DISCUSSION

The objective of these studies was to seek evidence of previous parasitization in unparasitized RBCs in acute falciparum malaria. The parasite-derived antigen Pf 155 or RESA[8] was chosen because it is associated with dense granules in the apical part of the merozoite and is deposited in the erythrocyte membrane during invasion.[9,10] RESA is, therefore, present in the RBC membrane from a very early stage of infection and acts as a ''footprint'' of RBC parasitization by a *P falciparum* parasite. RESA is a well-characterized polypeptide antigen that is highly conserved and is immunogenic in man.[11] Immune sera and MoAb 3362 gave a distinc-

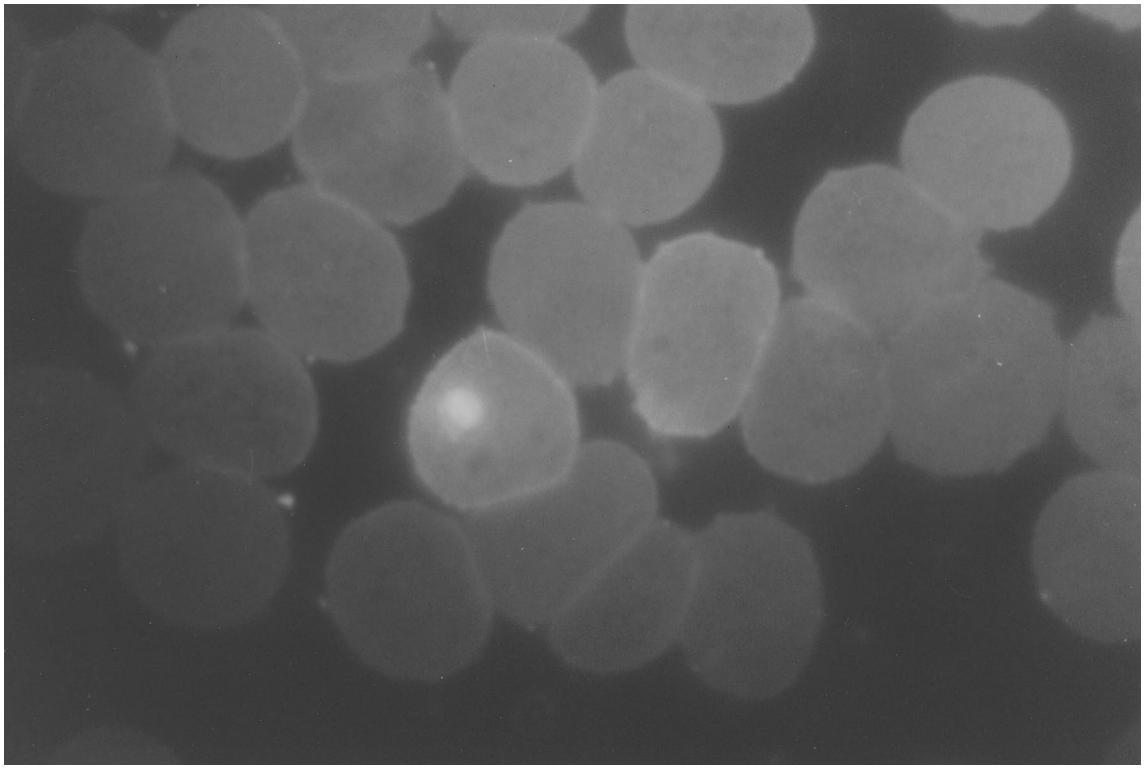

Fig 1. Immunofluorescence photomicrograph (original magnification × 1,000) stained with ethidium bromide and FITC-conjugated anti-RESA demonstrating a parasitized RBC on the left and a RESA positive, parasite negative RBC on the right.

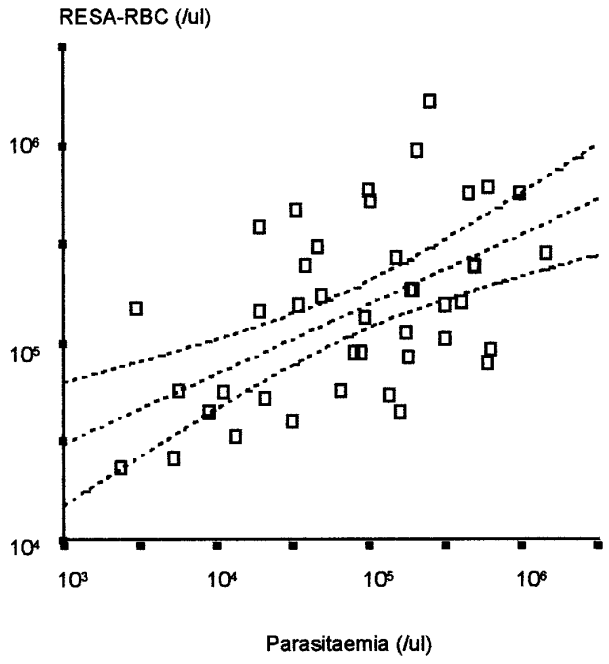

Fig 2. Linear regression analysis of parasitemia with the number of RESA positive, parasite negative RBCs (RESA-RBC) (mean, 95% confidence interval for the slope).

tive pattern of rim fluorescence over the glutaraldehyde-fixed erythrocyte membrane, but not on unfixed cells.[6] RESA is first synthesized within mature trophozoites and accumulates within merozoites. When RBCs from acute malaria patients were stained with pooled immune serum or MoAb3362, the pattern of antibody binding on some of the unparasitized RBCs was typical of RESA staining. Pooled immune serum is likely to have recognized a number of different parasite antigens, but the relative immunodominance of RESA was such that the two patterns of staining appeared identical.

There are three possible explanations for these observations. First, that in some cases, the merozoites invade the RBCs, then die spontaneously and are degraded rapidly within the cytoplasm. Second, that the young parasites are extruded actively by the RBC (either alive or dead), and third, that the parasites are somehow extracted from the RBC leaving it intact. If the parasites had simply died, then some DNA degradation products would still be expected to have stained by the ethidium bromide. This was not observed. The discrepancy between the numbers of RESA positive, parasite negative cells seen in vivo and in vitro suggests that active extrusion of parasites by the RBCs is unlikely to be quantitatively important. The most likely explanation is that host defense responses (probably the spleen) are involved in removal of intraerythrocytic parasites in vivo.[12-15] The spleen normally removes residual host nuclear material from erythrocytes, but how it recognizes damaged intraerythrocytic parasites is not known.

The possibility that immature or killed intraerythrocytic

parasites could be removed from within RBCs either by phagocytic cells or by active extrusion without their destruction, was first raised by observations in experimental simian malaria by Conrad and Dennis.[16] This was further supported by ultrastuctural studies of the spleen in Rhesus monkeys infected with *P knowlesi*.[16-19] Human neutrophils in vitro have also been shown to be capable of extracting *P falciparum* parasites from RBCs, leaving the RBCs intact[20] and parasitized RBCs treated with antimalarial drugs have been recorded extruding dead trophozoites.[21] This evidence in experimental systems supports our findings that removal of intraerythrocytic parasites occurs naturally in vivo when the host immune system can act, but not under in vitro parasite culture conditions in the absence of leukocytes and the spleen. It is likely that the parasites removed were in the first 24 hours of their 48-hour asexual life cycle because the morphology of the RESA positive cells was normal. In the second half of the asexual life cycle, cytoadherence takes place, and these adherent RBCs would have remained sequestered in the microcirculation whether or not they were still parasitized.[22] As most of the intraerythrocytic hemoglobin is consumed by more mature parasites, the hemoglobin concentration of the once parasitized cells was probably not reduced markedly. Thus, in the short-term, these once parasitized and antigenically marked RBCs, which occur in numbers approximately similar to the parasitized RBCs, will contribute to oxygen carriage and delivery, but their ultimate fate is not known. RESA is not expressed on the cell surface,[23] so increased antibody binding and thus immune recognition would not be expected, but it is associated with the RBC cytoskeleton[24] (through binding with spectrin) and could theoretically alter RBC deformability leading to splenic removal. Further study of the survival and the effects of different antimalarial drugs on the numbers of these cells in the circulation will be required.

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