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Possible basis for membrane changes in nonparasitized erythrocytes of malaria-infected animals *

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Previous studies (Gupta et al. (1982) Nature 299, 259–261) have shown that nonparasitized erythrocytes of *Plasmodium knowlesi*-infected monkeys contain the procoagulant phospholipid phosphatidylserine (PS) in the outer-half of their membrane bilayer. A reinvestigation of this problem has now revealed that in acute *P. knowlesi* infection, at least 30% of the infected animals do not have this abnormality. However, PS externalization was a consistent feature in the uninfected red cells of chronically infected animals. Also, a similar membrane change was observed in the red cells of uninfected splenectomized monkeys. These results strongly suggest that spleen plays an important role in maintaining the exclusive inner distribution of PS in the normal erythrocyte membrane, and that partial migration of this lipid to the outer monolayer in nonparasitized erythrocytes could be attributed to an abnormal physiology of this organ in malarial infection.

Blood stage malaria parasite develops and multiplies within the red cells of the infected host. The intracellular parasite produces several structural and functional alterations in the host erythrocyte membrane [1], presumably to facilitate its entry and subsequent growth within the cells. Besides the parasitized cells, nonparasitized erythrocytes of the malaria-infected animals also become abnormal [2–5]. These cells have a modified shape [3], reduced sialic acid content [4] and an altered membrane phospholipid organization [5]. However, the factors that induce these membrane changes in the erythrocytes are not yet known.

In an attempt to understand these factors, we analysed the transbilayer phospholipid distributions in nonparasitized red cells of the monkeys which had acute or chronic infections of *Plasmodium knowlesi*. Also, similar studies were undertaken on the red cells of uninfected or infected splenectomized animals. The data presented in this report, strongly suggest that membrane abnormalities in the nonparasitized erythrocytes could arise from an abnormal physiology of the spleen in malarial infections.

Synchronous acute infections of *P. knowlesi* were maintained by serial passage of infected blood in healthy rhesus monkeys (average weight 6 ± 1 kg), caged in a room illuminated with fluorescent light from 7:00 a.m. to 7:00 p.m. [6]. The monkeys were bled at the schizont stage and when parasitemia was 5–45%. Chronic infections of *P. knowlesi* (0.1–0.7%) were established by treating the infected monkeys at about 6% parasitemia with subcurative doses of chloroquine (5 mg/kg, 3 days). Normal healthy monkeys were also given chloroquine in identical conditions. Spleen from healthy monkeys were removed aseptically. The splenectomized animals were administered oxytetracycline (100 mg/day) intramuscularly for

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five days. Blood from these animals was drawn for experiments one month after the splenectomy.

Blood from normal, splenectomized or infected monkeys was drawn in heparinized glass tubes, and washed thrice with phosphate-buffered saline (pH 7.2). Leukocytes and platelets from the uninfected blood and leukocytes, platelets and schizonts from the infected blood were removed by means of a Ficoll-Conray gradient [7]. The nonparasitized erythrocytes thus obtained were contaminated with 1% red cells that were infected with early ring stage of *P. knowlesi,* as determined by Giemsa staining.

The transbilayer phospholipid distribution in erythrocytes was determined by means of bee venom and porcine pancreatic phospholipases A_2 (Sigma Chemical Company) as the external membrane probes [8]. Treatments of the cells with the enzymes were carried out for 60 min at 37°C, as described earlier [9,10]. Hemolysis was < 2%. Lipids were extracted from the whole cells [10], and the amount of phospholipid degradation determined by the known procedure [6]. Unsealed erythrocyte ghosts [11] were also subjected to enzyme hydrolysis in identical conditions.

Our earlier studies [5] have shown that nonparasitized red cells of monkeys that had acute *P. knowlesi* infections contain higher amounts of aminophospholipids in the outer-half of their membrane bilayer, as compared with the normal uninfected cells. Recently, we reinvestigated this problem in a large group of animals ($n = 20$, using been venom and porcine pancreatic phospholipases A_2 as the enzymatic probes. During these studies, we observed that the enzymes could hydrolyze 5–14% phosphatidylserine (PS) in the nonparasitized cells of only 70% of the infected animals. The remaining monkeys had a normal membrane lipid distribution in these erythrocytes. This suggests that the observed membrane changes in the nonparasitized red cells are not directly related to the presence of malarial parasite in the blood but could arise from some secondary complications.

The major secondary complication that occurs in malaria is known to be the histopathological alterations in the spleen, leading in some cases to splenomegaly [12]. It may, therefore, be considered that if an abnormal spleen physiology is responsible for inducing membrane changes in the nonparasitized erythrocytes of acutely infected monkeys, then these cells from chronically infected animals should consistently have an abnormal membrane lipid organization [13]. To examine this possibility, we analysed the transbilayer phospholipid distributions in the nonparasitized red cells of chronically infected monkeys. Table I shows that unlike the control cells, both bee venom and pancreatic phospholipases A_2 hydrolysed PS in the intact nonparasitized erythrocytes. This finding was consistent in all the blood samples analysed, suggesting that PS externalization in the nonparasitized cells may be related with malaria-induced physiological changes in the spleen.

To confirm that abnormal physiology of the

TABLE I

PHOSPHOLIPASE A_2-CATALYSED HYDROLYSIS OF MEMBRANE PHOSPHOLIPIDS IN NONPARASITIZED ERYTHROCYTES OF MONKEYS CHRONICALLY INFECTED WITH *P. KNOWLESI*

Values shown are the mean of three determinations each on blood samples of eight animals ± S.D. Control cells are the erythrocytes of normal healthy monkeys that were given chloroquine as described in the text.

Sample	Source of enzyme	PC (%)	PE (%)	PS (%)
Control cells	Bee venom	46.3 ± 3.8	21.7 ± 1.4	0
	Porcine pancreas	5.7 ± 2.2	0	0
Nonparasitized cells	Bee venom	39.3 ± 1.5	23.8 ± 1.1	10.2 ± 2.0
	Porcine pancreas	19.8 ± 2.4	13.8 ± 4.0	9.9 ± 2.5
Unsealed ghosts	Bee venom	100	100	100
	Porcine pancreas	100	100	100

TABLE II

PHOSPHOLIPASE A_2-CATALYSED HYDROLYSIS OF MEMBRANE PHOSPHOLIPIDS IN SPLENECTOMIZED MONKEY RED CELLS

Values shown are mean of three determinations each on blood samples of four animals ± S.D.

Sample	Source of enzyme	PC (%)	PE (%)	PS (%)
Normal monkey cells	Bee venom	47.1 ± 1.1	18.3 ± 2.2	0
	Porcine pancreas	6.2 ± 2.5	0	0
Splenectomized monkey cells	Bee venom	43.5 ± 3.9	20.2 ± 2.9	13.4 ± 2.5
	Porcine pancreas	15.0 ± 3.2	9.8 ± 1.8	10.6 ± 1.6
Unsealed ghosts	Bee venom	100	100	100
	Porcine pancreas	100	100	100

spleen during malaria could lead to membrane abnormalities in the nonparasitized erythrocytes, we analysed the membrane lipid distribution in the red cells of splenectomized monkeys. Table II shows that PS was not hydrolysed by phospholipases A_2 in the intact red cells of normal healthy monkeys, but after splenectomising the same group of animals, these enzymes readily degraded 9–16% of this phospholipid. To examine whether this accessibility of PS to phospholipases is further enhanced by infecting these splenectomized monkeys with *P. knowlesi*, we determined the extent of phospholipid degradation in the nonparasitized red cells of these animals. The amounts of hydrolyzed phosphatidylethanolamine (PE) and PS were 12–20% and 11–18%, respectively, suggesting that the membrane phospholipid organization in the uninfected erythrocytes of splenectomized monkeys is not influenced by the malarial infection.

Spleen is the major organ responsible for destruction of old red blood cells [14]. It may, therefore, be argued that removal of this organ may result in an accumulation of the old cells, and hence the PS externalization in erythrocytes of splenectomized animals [15]. But this does not seem to be the case, as it has been shown that the red survival remains unaffected by the removal of the spleen [16].

This study clearly indicates that membrane abnormalities in nonparasitized red cells of malaria-infected animals possibly arise from histopathological alterations in the spleen. As the extent of these alterations should vary with the animal, depending mainly on the physiological status of the organ, we conclude that only animals with physiologically weak spleen could have membrane abnormalities in their nonparasitized erythrocytes during acute *P. knowlesi* infections.

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