# A new look at nonparasitized red cells of malaria-infected monkeys

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**Many reports have shown that malarial parasites can produce distinct morphological and molecular alterations in the membranes of the parasitized erythrocytes[1–8], but few studies have been carried out on nonparasitized erythrocytes of infected animals[9–11]. We report here that the outer leaflet of the membrane bilayer of nonparasitized erythrocytes contains significantly larger amounts of aminophospholipids (phosphatidylethanolamine (PE) and phosphatidylserine (PS)), than the normal red cell membrane. This alteration in nonparasitized red cells is probably caused by $Ca^{2+}$-induced cross-linking of spectrin, and gradually disappears after chloroquine treatment. The external localization of PS in these cells together with defective structure of their cytoskeletal network provide a strong basis for the complications associated with malaria infection like thrombosis, infarction and severe anaemia.**

The transbilayer distributions of the diacylglycerophospholipids, phosphatidylcholine (PC), PE and PS, in the membranes of normal erythrocytes of uninfected monkeys as well as nonparasitized red cells of *Plasmodium knowlesi*-infected monkeys were investigated using phospholipase $A_2$ and 2,4,6-trinitrobenzenesulphonic acid (TNBS) as external membrane probes[12,13]. The results shown in Table 1 reveal marked differences between the two types of cells after treatments with phospholipase $A_2$. Unlike the normal intact cells, PE (~31%) and PS (~15%) in nonparasitized erythrocytes from infected monkeys were readily accessible to the enzyme. Almost complete (92–100%) hydrolysis of all the glycerophospholipids occurred when unsealed ghosts of the normal and nonparasitized cells were separately treated, in identical conditions, with phospholipase $A_2$.

Treatments of both types of cells with TNBS also reflected notable differences (Table 2). In the nonparasitized cells, TNBS modified both PE (~35%) and PS (~16%) whereas in the normal cells, only PE (~19%) was labelled by the reagent. The increased amounts of PE in the outer surface of the nonparasitized cells, as compared with the normal cells, did not arise from the transmembrane movement[14] of this lipid from the inner to the outer surface of the cell membrane because almost equal amounts of PE were labelled when the cells were treated with TNBS for 4, 6 and 10 h. Also, the observed differences in the amounts of labelled aminophospholipids were not due to a partial reaction with reagents, as PE and PS were completely labelled when unsealed ghosts of the two types of cells were reacted with TNBS in identical conditions. Attempts were also made to use these methods to study the nonparasitized cells obtained from blood of monkeys which developed >30% parasitaemia. Invariably, extensive haemolysis of cells occurred on

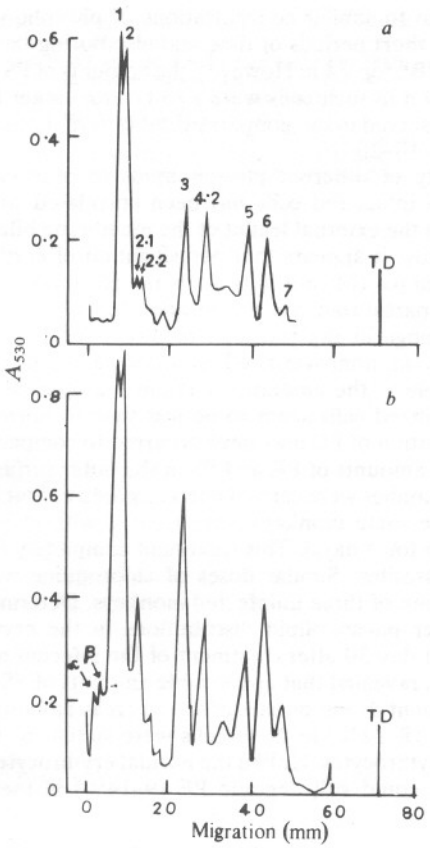


**Fig. 1** Densitometer scans of gels stained for proteins with Coomassie blue after electrophoresis of red cell ghosts on polyacrylamide gels in the presence of SDS (ref. 22). *a*, Normal monkey erythrocyte ghosts; *b*, ghosts of nonparasitized erythrocytes of *P. knowlesi*-infected monkey (parasitaemia ~12%). The protein bands of the normal monkey erythrocyte are numbered according to the nomenclature of Fairbanks *et al.*[22]. TD, tracking dye.

**Table 1** Erythrocyte phospholipid degradation by phospholipase $A_2$

| Sample | $n$* | PC (%) | PE (%) | PS (%) |
|---|---|---|---|---|
| Normal red cells | 15 | 57.35±4.11 | 0 | 0 |
| Nonparasitized red cells | 12 | 48.31±4.23 | 31.31±2.40 | 15.19±2.32 |

Synchronous infections of *Plasmodium knowlesi* (WI strain) were maintained in healthy rhesus monkeys as described earlier[8]. The monkeys were bled when parasites were at the schizont stage. Blood was drawn into heparinized glass tubes and washed three times with phosphate-buffered saline (*p*H 7.2). Leukocytes and platelets from normal blood and leukocytes, platelets and schizonts from infected blood were removed by means of a Ficoll–Hypaque gradient[26]. The nonparasitized red cells thus obtained were invariably contaminated with <1% erythrocytes that were infected with early ring stage of *P. knowlesi*, as determined by Giemsa staining. Erythrocytes were treated with phospholipase $A_2$ (purified from *Naja naja* snake venom, Haeffkin Institute, Bombay) essentially in the conditions described earlier[8]. After inhibiting the enzyme activity by addition of *o*-phenanthroline and EDTA[27], lipids were extracted from cells. The lipid mixture was chromatographed on silica gel G-60 TLC plates as described previously[8] and the total phosphorus content in each spot was determined[28]. The percentage of the total phospholipid hydrolysed after treatment of red cells with the enzyme was determined by measuring the ratio of remaining diacylglycerophospholipids to the corresponding lyso derivative. Values are expressed as mean±s.d. <5% cells were lysed under the experimental conditions. Unsealed ghosts, prepared[29] from both the normal and nonparasitized cells, were also treated with the enzyme in identical conditions. Almost complete (92–98%) hydrolysis of all the diacylglycerophospholipids was observed in both the cases. *n*, Number of determinations.

* Three determinations on each blood sample of five uninfected monkeys and four infected monkeys (parasitaemia 10–20%).

**Table 2** Labelling of erythrocyte aminophospholipids with TNBS

| Sample | $n$* | PE (%) | PS(%) |
|---|---|---|---|
| Normal red cells | 12 | 18.55±2.87 | 0 |
| Nonparasitized red cells | 9 | 34.89±2.81 | 16.10±2.43 |

Labelling of erythrocytes with TNBS was carried out according to the reported procedure[30]. The incubations were done at 20°±2 °C for 4, 6 and 10 h. No differences in the percentage of labelling in these experiments were observed. No detectable cell lysis occurred in all these experiments. The percentage of TNBS labelling was determined by the total phosphorus determinations and also by measuring absorbance of yellow colour at 340 nm. Values shown are mean± s,d.. All the aminophospholipids were completely labelled when unsealed membrane ghosts from both the normal and nonparasitized cells were treated with TNBS under identical conditions. *n*, Number of determinations.

* Three determinations on each blood sample of four uninfected monkeys and three infected monkeys (parasitaemia 10–20%).

exposing them to similar concentrations of phospholipase $A_2$ for relatively short periods of time and also during their treatment with TNBS for >4 h. However, the amounts of PS labelled by TNBS in 4 h in such cells were significantly larger (~40%) than those observed in the nonparasitized cells of monkeys with parasitaemia (10–20%).

Accessibility of different phospholipids to phospholipases and TNBS in intact red cells has been correlated with their localization in the external leaflet of the membrane bilayer[11,12]. From this study, it appears that parasitization of erythrocytes by the malarial parasite not only alters the phospholipid asymmetry in the parasitized cell membrane[8], but also leads to an enormous change in the transbilayer distributions of various phospholipids in nonparasitized erythrocyte membrane. As shown in Table 1, the amounts of PC in the outer surface of the nonparasitized cells seem to be less than in normal cells. This redistribution of PC may have occurred to compensate for the increased amounts of PE and PS in the outer surface.

The above studies were carried out on a group of four infected monkeys. The same monkeys were treated with chloroquine (20 mg per kg for 3 days). This treatment completely removed circulating parasites. Similar doses of chloroquine were also given to a group of three uninfected monkeys. Determinations of transbilayer phospholipid distributions in the erythrocyte membrane on day 30 after treatment of the infected monkeys with the drug, revealed that appreciable amounts of PS (5–8%) were still present in the outer surface whereas amounts of the external PE (18–22%) in these cells were similar to those of the normal erythrocytes. Unlike the normal erythrocytes, phospholipase $A_2$ could still degrade PE (9–14%) in these cells.

**Table 3** Concentrations of GSH in erythrocytes

| Sample | *n* | GSH (μmol per ml packed cells) |
|---|---|---|
| Normal red cells | 10 | 2.2±0.7 |
| Nonparasitized red cells* | 6 | 1.9±0.3 |

Concentrations of GSH in red cells were determined by the procedure of Beutler *et al.*[31]. Values are expressed as mean±s.d.; *n*, number of animals.
* Parasitaemia 10–20%.

However, by day 60, the amounts of PE (16–20%) and PS (none) in the external surface were normal. Chloroquine treatments of uninfected monkeys had no effect on red cell membrane phospholipid asymmetry.

Recent studies have shown that strong interactions between aminophospholipids and cytoskeletal proteins (specifically spectrin) probably determine phospholipid asymmetry in the erythrocyte membrane[15–17]. This asymmetry in red cells is lost if the spectrin component of the cytoskeletal network becomes defective[14,15]. Defects in the structure of spectrin may originate from decreased concentrations of glutathione (GSH) or from elevated levels of $Ca^{2+}$ in cells[18–21]. To look at this, membrane proteins of nonparasitized red cells were analysed by SDS-polyacrylamide gel electrophoresis[22]. Similar studies were also carried out on normal red cells of a group of six uninfected monkeys. The gel profiles of membrane proteins of nonparasitized erythrocytes from five different monkeys (parasitaemia 10–20%) invariably showed two additional high molecular weight protein bands ($\alpha$ and $\beta$, Fig. 1*b*) of variable intensity, which were absent in the gel profiles of the normal cell ghosts (Fig. 1*a*). No correlation was found between the intensity of the two bands and the extent of parasitaemia in monkeys. From a comparison between the relative intensities of protein bands present in the gel profiles of normal and nonparasitized erythrocyte ghosts, it appears that the two protein bands arise from cross-linking of spectrin. To examine whether these bands originate from oxidation of sulphydryl groups of the protein[15], the nonparasitized cell ghosts were incubated with dithiothreitol at 37 °C for 60 min before their extraction. This treatment had

**Table 4** $Ca^{2+}$ contents of normal red cells of uninfected monkeys and nonparasitized erythrocytes of *P. knowlesi*-infected monkeys

| Sample | *n* | $Ca^{2+}$ (μg per ml packed cells) |
|---|---|---|
| Normal red cells | 5 | 0.61±0.39 |
| Nonparasitized red cells* | 6 | 2.36±1.67 |

$Ca^{2+}$ content in red cells was determined by atomic absorption spectrophotometry as described by Harrison and Long[32].
* Parasitaemia 10–20%.

no effect on these bands, implying that the two high molecular weight proteins did not arise from oxidation of sulphydryl groups of spectrin. This is further supported by our finding that almost equal concentrations of GSH were present in normal as well as nonparasitized cells (Table 3). Nevertheless, the $Ca^{2+}$ levels in the two types of cells were significantly different (Table 4). The elevated levels of $Ca^{2+}$ present in the nonparasitized cells are compatible with earlier findings that malaria infection in animals results in diminished levels of ATPase activity in parasitized as well as nonparasitized red cells[10]. A partial (or complete) deactivation of ATPase in these cells would result in the elevated levels of $Ca^{2+}$ and consequently activation of transglutaminase which may induce cross-linking of spectrin in the nonparasitized cells[18–20].

Normal structure of spectrin seems essential for the invasion of red cells by the malarial parasite[23]. This means that if the structure of this protein becomes defective in the total population of nonparasitized erythrocytes in a malaria-infected animal then further invasion would not proceed. That parasitaemia does regularly increase in the infected monkeys suggests that only a fraction of the total population of the nonparasitized cells becomes abnormal. This conclusion is supported by the observation that unlike the nonparasitized cells, erythrocytes containing malarial parasite at the early ring stage have PS located exclusively in the inner leaflet of the membrane bilayer[8].

Previous findings[9,10] indicate that the serum of malaria-infected animals contains some unknown factors which induce changes in the nonparasitized cells. We, therefore, incubated normal erythrocytes with serum of heavily infected monkeys at 37 °C for 2–3 h. Treatments of such cells with TNBS and phospholipase have so far not revealed any effect of the serum but further studies are in progress.

These observed abnormalities suggest that externalization of PS in these cells could enhance blood coagulation[24,25] and, therefore, may cause thrombosis. Also these cells, because of their defective cytoskeletal network, would be rapidly cleared from the circulation by the spleen[18], making the animals more anaemic.

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