J Vect Borne Dis 44, March 2007, pp. 23–32

Study of *P. falciparum*-infected erythrocytes and induced anisotropies under optical and fluid forces

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Abstract

Background & objectives: The effect of *P. falciparum* on erythrocytes has been studied for a long time at the population level but actual studies at the single cell level remain largely unexplored. The aim of this study was to address the host-parasite relationship at the single cell level under two different kinds of forces, an optical force and a fluid force. The questions addressed were about the basic host-parasite interactions, but our findings have larger implications in diverse fields of parasite biology.

Methods: Erythrocytes were monitored under optical forces (using optical tweezers) and fluid forces (using microfluidic chambers) and dynamical images were captured in real-time video clips. These videos were then split into their respective frames so as to yield temporal information and various parameters pertaining to membrane structure, ionic imbalance and interaction with different forces were studied.

Results: The results of this study mainly bring to fore the inherent differences between infected and normal cell populations at the single cell level under various external forces. We probed three different criteria folding times, rotation speeds and rolling frequency to show inherent difference in various cell populations and also the dependence of the above to the cycle of the parasite.

Interpretation & conclusion: This study portrays the importance of single cell observations pertaining to the host-parasite relationship. It shows the effect the malarial parasite has on erythrocytes and how the intrinsic property of the infected and its neighbouring uninfected cells change as compared to normal erythrocytes. There are thus implications in the fields of cytoadherence, parasite invasions and host immune evasion.

Key words Cytoadherence – flow cell – malaria-infected red blood cells – optical laser tweezer – red blood cells (RBC) – rolling

Introduction

Malaria affects approximately 300 million people worldwide, and about 1.5 million of these die every year in the tropics. *Plasmodium falciparum* malaria is the most pathogenic, since it can lead to fatal cerebral malaria. We have studied population of normal erythrocytes and *P. falciparum*-infected erythrocytes under optical and fluid forces: two distinct forces, but similar in their order of magnitudes. The optical

forces were applied using an optical tweezers setup and the fluid forces in a microfluidic chamber, both apparatus being indigenously designed and fabricated. Both these studies point out differences between the two populations in terms of various anisotropies (ionic, mass, shape) induced by the parasite. These studies afford new insights into the changes brought about by the parasite in a stage specific manner and into cell non-autonomous effects mediated by the parasite.

Studies under optical forces: The ability of optical tweezers[1] to trap and manipulate single cells has opened new vistas for basic research in the life sciences, like single cell molecular biology[2], as well as for diverse applications ranging from laser-assisted *in vitro* fertilization[3] and the development of cell biosensors[4] to new possibilities of micromanipulation of relevance to cell sorting and cellular microchips[5,6]. New insights have been forthcoming from application of optical tweezers to studies of the mechanics of single DNA molecules[7]. Optical trapping also makes feasible single-cell testing of erythrocytes that are linked to pharmacophores for use in drug therapy[8].

This study of erythrocyte population stemmed from a simple observation made, that showed very basic differences in behaviour of uninfected and malaria-infected erythrocyte populations within the optical trap. The normal biconcave disc shaped erythrocytes underwent a deformation to form a rod like structure that aligned itself to along the electrical field axis. Infected erythrocyte in the linearly polarised light not only undergoes shape deformation but also rotates in a plane perpendicular to the laser propagation direction[9]. Normal erythrocytes could also be made to rotate under circular polarised light. We have also reported that these rotation periods depend on the size of the cell and the laser power used[10]. The folding action of a trapped cell, be it infected or healthy, has recently been successfully modeled by us using the concept of Euler buckling instability[11].

Studies under fluid forces: The pathogenicity of *Plasmodium falciparum* results from its unique ability to adhere to endothelium and uninfected erythrocytes. It is, therefore, important to understand the events leading to flowing blood cells undergoing such adhesion. Largely based on the leukocyte adhesion model, it is postulated that the slowing down (rolling) of *Plasmodium*-infected red blood cells (PRBCs) is initiated by interactions between certain host adhesion molecules and the parasite proteins. In this article we present data demonstrating that PRBCs do not require the presence of host adhesion molecules to slow down and roll. In a synchronised culture, the proportion of slow-flowing cells increased with parasite development and was highest at the trophozoite stage. We also observed that the uninfected red blood cells (URBCs), originating from a parasite culture containing PRBCs, were also inherently slower compared to malaria-unexposed normal red blood cells (NRBCs). NRBCs became slower upon incubation with supernatant taken from a parasite culture. However, such an effect was transient and the NRBCs reverted to their normal flow speed within 12 h upon withdrawal of culture supernatant. Based on our observations, we suggest that the higher propensity of PRBCs and URBCs to slow down is due to inherent structural anisotropy and altered membrane rigidity. Thus the initial events leading to the slowing down of malaria-infected blood cells appear to be different from those occurring during leukocyte adhesion.

These studies together implicate the role of the parasite in mediating membrane changes and also bring into focus different ion channels that are modified by the parasite and their downstream effects in mediating apoptosis like processes via cell non-autonomous process[12].

Material & Methods

Optical tweezers: The optical tweezers setup used was fabricated in our laboratory[13]. Trapping was achieved using a linearly polarised light in single

beam optical tweezers geometry. A strongly focused laser beam was used to generate gradient forces, F_{grad} that pulls dielectric particle towards the focal zone with magnitudes larger than the scattering forces, F_{scat} that pushes the particle along the incident light direction (Fig. 1a).

Flow-cell: The flow-cell was constructed by using a glass slide of dimensions 75 × 25 × 1 mm and a cover slip of dimensions 60 × 22 × 0.17 mm, with 0.4 mm thick spacers in between to form the flow channel[12]. The effective inner dimensions of the flow channel were 35 × 10 × 0.4 mm. A peristaltic pump (Pharmacia LKB-Pump P-1) was used to maintain continuous flow of the red cell suspension through the chamber at a constant flow of 1.4 ml/min (Fig. 1b). Before running in the flow-cell, cells were pelleted at 3000 rpm for 10 min and resuspended in RPMI 1640 with 0.5% albumax. Cells were used at a concentration of 10^6 cells/ml.

Sample preparation: Asexual stages of *Plasmodium falciparum* 3D7 strain were maintained *in vitro* in 5% hematocrit in RPMI 1640 medium containing 0.5% albumax and 80 µg/ml gentamycin sulphate in a humidified chamber containing 5% CO_2 at 37 ºC in human erythrocytes of serological type O+ as described earlier[14]. To obtain synchronised cultures, sorbitol treatment according to the method described earlier[15] was performed.

The *P. berghei* ANKA strain was maintained by passaging asexual stages through BALB/c mice. Tail bleeds were collected from mice showing approximately 20–40% peripheral blood parasitaemia, and diluted 1 : 10 in incomplete RPMI. About 100 ml of this dilution was injected intraperitoneally into naïve BALB/c mice for the expansion of parasites. Parasitaemia was monitored by microscopic examination of blood smears stained with Giemsa. Blood samples from humans and mice were collected in a sterile tube containing the anticoagulant ACD (136 mM glucose; 38 mM citric acid monohydrate and 75 mM sodium citrate) and centrifused at 3000 rpm for 10 min. The plasma and buffy coat containing white blood cells were aspirated out and purified erythrocytes were washed with sterile RPMI (Life Technologies, Inc) medium with 28 mM $NaHCO_3$, 25 mM HEPES and 80 µg/ml gentamycin sulphate. The erythrocytes were resuspended in sterile RPMI medium containing 0.5% albumax (Life Technologies, Inc) as a 50% v/v suspension, and diluted appropriately. Before imaging, RBCs were pelleted at 3000 rpm for 10 min, resuspended in PBS solution composed of NaCl (136 mM), KCl (2.68 mM), Na_2HPO_4 (10.14 mM), and KH_2PO_4 with 2% BSA or complete RPMI medium at a dilution of about 10^6 cells/ml, from which 40–50 µl was used on a microscope slide with a cover slip for imaging and trapping. Nomenclature used for different popula-

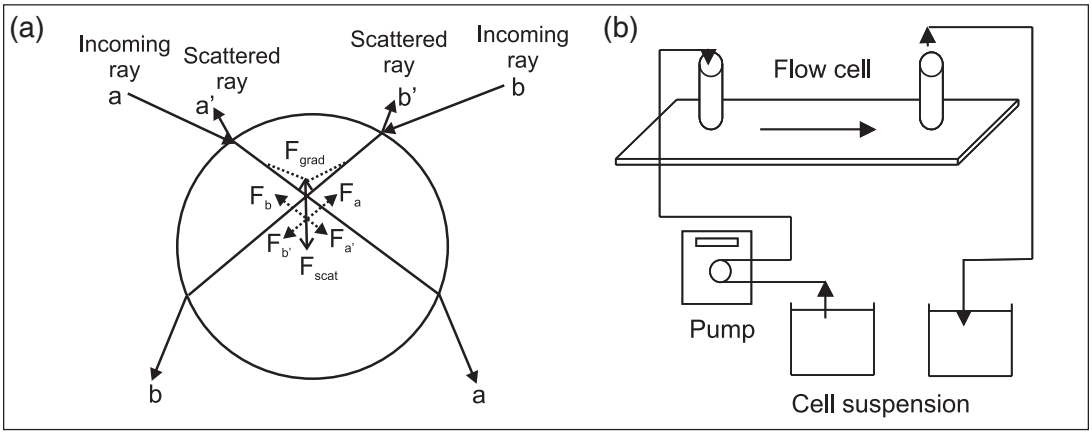

Fig. 1: (a) Ray diagram showing the gradient force and scattering force due to laser beam focused on a dielectric particle. Trapping is achieved when the gradient forces (F_{grad}) are more than the scattering forces (F_{scat}); and (b) The microfluidic setup used in the flow experiments

tions of red cells is as follows: PRBCs, *Plasmodium*-infected red blood cells; URBCs, uninfected red blood cells exposed to PRBCs in a parasitised blood sample; IRBCs, red cells of a parasitised blood sample, a population containing both PRBCs and URBCs; and NRBC, malaria-unexposed normal red blood cells. For each set of measurements for *P. falciparum*, the same source of red blood cells was used for the preparation of NRBC and IRBC, and the cells were taken through identical treatments of incubation in a humidified chamber containing 5% CO_2 at 37ºC. In case of *P. berghei*-containing samples, NRBCs were obtained from uninfected and IRBCs from infected mice.

Measurements and quantitation: The coverslip/flow-cell was mounted on an inverted microscope (Nikon TE200-U) and imaged through a 100X oil immersion objective using a CCD camera interfaced to a laboratory computer. Real-time videos of cells were monitored frame by frame, and their velocities, speeds and folding times were measured. The time interval between two consecutive frames was 40 ms. The speed of a flowing cell was deduced from the number of video frames occupied by the cell in the field of view. Cells moving at < 400 μm/sec were counted as rolling cells. For each point of measurement, the speed of at least 100 cells was measured. The speeds of rotation were measured by counting the number of frames a cell took to complete one rotation. Folding and unfolding times were measured as the number of frames it took a cell to completely undergo a change in shape—from a disc to a rod like shape or *vice versa*.

Results

Trapping of erythrocytes: Erythrocytes are biconcave discs about 7 μm in diam and 2 μm in thickness at the edges. When an erythrocyte comes to the focus of the optical trap it undergoes a shape change. The biconcave disc buckles to collapse into a rod-like shape (Fig. 2a). The time scale for this process of folding is of the order of 200 ms to 1 sec, depending upon incident laser power and cell size. When the laser is blocked the cells relax and slowly unfold back to their normal biconcave disc shape. The time scales for unfolding are of the order of magnitude of 10 to 20 sec[9,11]. Once the cells fold in the trap they align themselves along the electrical field vector of the laser light used. Folding of cells in the trap is shown to be directly proportional to the laser power used where for every cell there is a corresponding value of L^0 under which the cell does not fold[11]. Folding times of cells also depend on the viscosity of the suspending medium, the size of the cell, the stage of infection and the ionic composition of the suspending medium (Roy *et al*, unpublished data).

Rotation of erythrocytes: The infected erythrocytes showed an intrinsic difference in behaviour when compared to normal erythrocytes. After buckling and folding, the normal erythrocyte aligns itself to the electrical axis of the plane polarised light used. On the contrary the infected erythrocyte fold followed by a twist (Fig. 2b) before it rotates with linearly polarised beam. The speed of these rotations depends directly

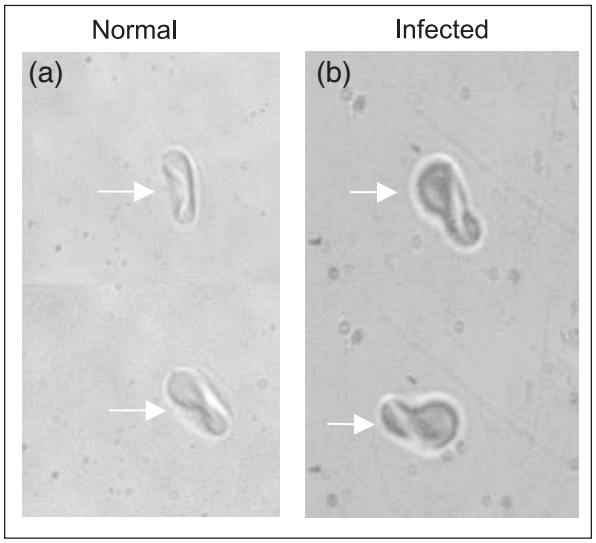

Fig. 2: (a) Shows two normal cells in a laser trap after folding; and (b) Shows two infected cells in a laser trap. The twisted structure of the infected cells is distinct in (b) while it is absent in the normal cells in (a)

on the size of the cell and the laser power used[10]. The speed of rotations of infected red cells also changed with the stage of infection peaking at the trophozoite stage (Roy *et al*, unpublished data). Normal erythrocytes could also be made to rotate using a circularly polarised light.

Rolling of erythrocytes: We exposed the red blood cells to fluid forces, in the absence of any protein/cell coating on the walls of the parallel plate glass flow-cell. Most of the cells were found to align (in streamline fashion) along the direction of flow of the fluid medium. However, a certain percentage showed distinct cartwheel type of rolling motion (Fig. 3). However, it is evident that rolling of red blood cells does not absolutely require endothelial cells or protein-protein interactions, but can be explained in terms of hydrodynamic fluid forces on the anisotropic red cells. What is even more important is that normal erythrocytes show rolling behaviour in the absence of endothelial cells, showing that a small population of normal erythrocytes at any given time have inbuilt anisotropies in them[12].

The distribution of speeds for population of NRBCs and IRBCs at different stages post-synchronisation is shown in Fig. 4a. It is apparent that within the population that is counted, a bulk of IRBCs moved slower than NRBCs, and this fraction was found to be largest at the trophozoite stage (22 h post-synchronisation). About 30% the NRBCs were found to roll, and this value increased about 50% for the 18–24 h post-synchronised IRBCs (trophozoite stage; Fig. 4b). The same assay was also used for the murine malarial parasite *P. berghei* and a two to three-fold higher number of rolling cells was observed amongst the asynchronous IRBCs obtained from infected mice. Thus we see that the infected populations of erythrocytes have a higher propensity to roll than normal populations. We also observe in a synchronous culture that at the trophozoite stage IRBCs mediate an increase in rolling of URBCs in that same population. These results have also been confirmed by using culture supernatant from a synchronised culture on normal erythrocytes. It has been observed that these normal erythrocytes also exhibit a higher rolling propensity after incubation with the culture supernatant. We have also seen that the frequency of rolling is inversely dependant on the temperature indicating the role of membrane rigidity in rolling[12].

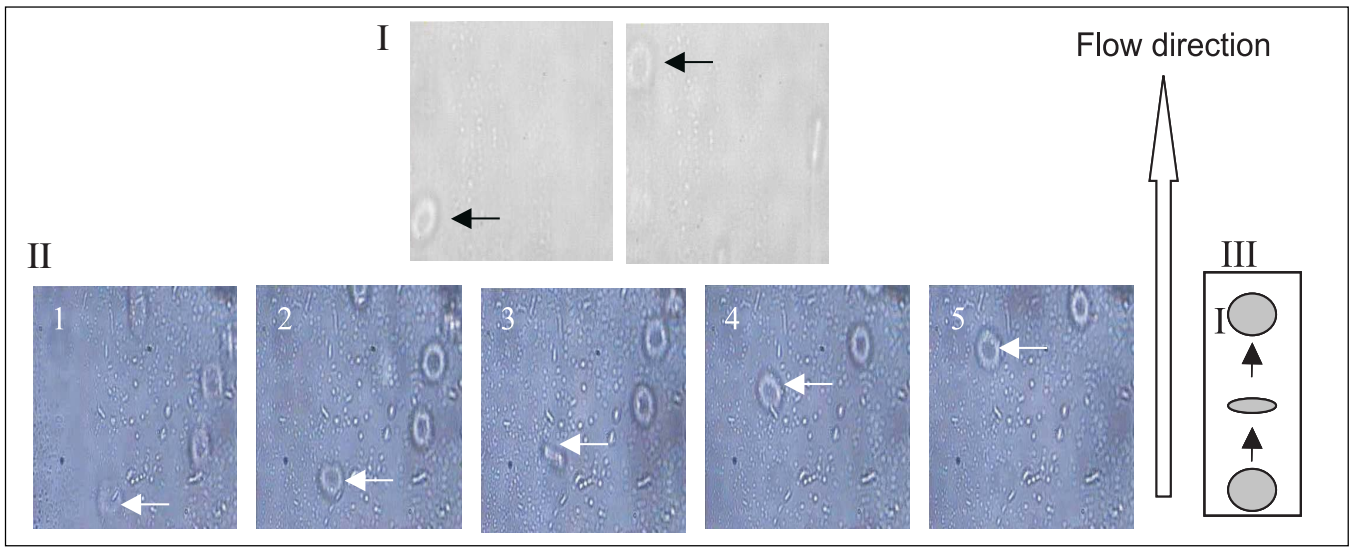

Fig. 3: Panel I shows a normal cell in two consecutive frames as marked by arrows (fast moving); the bottom panel (II) shows a rolling cell in five consecutive frames marked by arrows (slow moving); and Panel III shows a pictorial depiction of a rolling cell, the thick arrow shows the direction of fluid flow

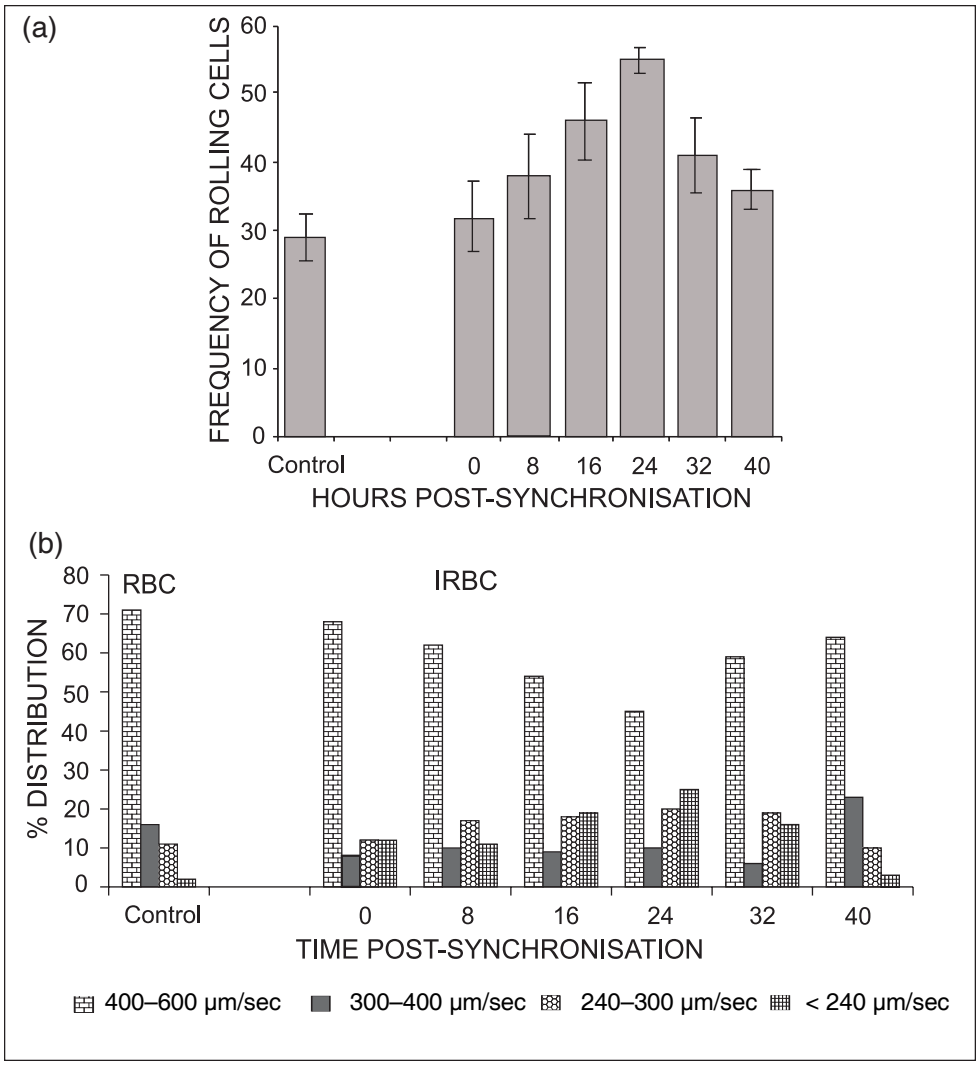

Fig. 4 : (a) Flux of rolling RBCs and IRBCs, measured as the fraction of red cells with a velocity of < 400 µm/sec; and (b) Velocity distribution of a population of normal blood cells (RBC) and synchronised *Plasmodium falciparum* infected RBCs (IRBC; parasitaemia 2.8%) as counted through video imaging[12]

Discussion

We have explained our results under the action of optical forces extensively in our earlier work[9]. In brief, when RBC is trapped using a linearly polarized light of electric field E, it induces a dipole moment given by the relation, $p = \alpha E$, α being the polarizability of RBC. For a parasite infected red cell, α, which is a tensor, is asymmetric. This asymmetry in the polarizability gives rise to light induced torque, which results in the alignment of the induced dipole along the electric field minimising the potential energy. For normal RBC, such asymmetry in the polarizability is essentially small and does not induce any rotational motion. Recently, it has been observed that the transport of ions is significantly altered upon infection of RBC by the parasite[16,17]. The increase in the asymmetry of the polarizability in case of infected RBC is likely to be due to difference in transport of various ionic species, which causes rotation of infected RBCs in linearly polarized light.

Parasite driven rolling has been demonstrated, depends on a minimum shear stress, although tethering and adhesion were observed at all shear values that were tested (0.5 to 3.0 dyn/cm^2). It was also observed in this study that more than half the cells that showed rolling did not adhere in the field of observation, and the authors concluded that the rolling of IRBCs might be the rate-limiting step in cytoadherence[18]. In earlier studies, observations have been made of rolling of erythrocytes at very high shear stress value[18,19]. In our experiment we are able to show cells rolling at much lower shear stress value. This large difference lies in the definition of the term rolling and the speed at which rolling is determined. In previous articles a cell was defined as rolling if it was moving at speeds lower than 150 μm/sec. Due to the use of a 100X objective and a fast camera we were able to drastically increase this cut-off to a value of 400 μm/sec. We also took measurements using the previously established cut off (150 μm/sec). We find that the number of RBCs that roll now increase to 25% from the 2% that we observed during the trophozoite stage. Using the dimension of the flow cell and the flow rates we calculated the shear pressure to be 0.9 dyn/cm^2 (0.09 Pa). By altering the flow rates we also checked the effect of shear pressure on rolling of erythrocytes. Lowering of the shear pressure up to four folds did not show any change in the frequency of rolling. This indicates that at such low shear pressure the cell shape and membrane rigidity determines the rolling of the cell more than the shear pressure exerted on it.

The rolling of parasite-infected and uninfected erythrocytes is determined by multiple parameters of which the primary determinant is the stage of the parasite during its intraerythrocytic life cycle. The frequency of cells that roll show a strong correlation with the development of the parasite within the host erythrocyte, increasing till the trophozoite stage after which the frequency drops down. This shows that at the trophozoites stage the infected cells are inherently more prone to flow at a slower rate and thereby are more amenable to interaction with an array of host cells (endothelial, normal erythrocytes and platelets). It has been already reported *in vitro* that most 16 h old trophozoites are capable of adhesion and are found adhered to endothelial cells in culture systems[20].

Another important parameter that could be involved in rolling is the elasticity of the membranes. It has been previously reported that the elasticity of the membrane has a strong correlation with temperature. It has been shown that at lower temperatures the membrane is rigid. As the temperature is raised a phase transition takes place around 37ºC. We recorded the effect of membrane elasticity on rolling by manipulating the temperature of the fluid in which the cells were suspended[12]. We observed that as the temperature was lowered the cells showed a higher frequency of rolling. In these experiments the viscocity was kept constant thus implicating the role of membrane rigidity on rolling. In infected cells it has been shown that with the progression of the intraerythrocytic stage there is an increase in membrane rigidity. Microfluidic experiments have shown the inability of infected cells to traverse narrow channels[21]. The infected cells that traverse narrow channels have also been shown to have increasingly large times to recover its shape[22,23].

Most of these experiments were conducted using samples from parasite tissue culture systems. The average parasitaemia that was maintained for most of these experiments was around 3%. In an uncomplicated case of malaria the parasitaemia ranges anywhere from 0.1–1% and in many severe cases can go up to as high as 20%[24]. This shows that these experiments were carried out under physiological conditions. We also observe in our experiments that there are a large number of uninfected cells in the culture that show a high propensity to roll. Using ektacytometry it has been reported previously that uninfected red cells that were taken from malarial patients had much lower elasticity when compared to healthy human erythrocytes[25]. A highly debated ex-

planation for this decrease in this elasticity has been exo-antigens that are secreted by the infected cells. Earlier it was reported that this exo-antigens were heat sensitive[26] but later groups could not find any such heat sensitive component[27].

In our experiments we see that heat has no effect on the factors that cause the increased rolling of infected cells. The factors that had an effect in our experiments were also transient, i.e. they showed an effect within 12 h and the behaviour was reversible by treating the cells with normal medium. This has great implication in parts of the body other than capillaries, where cells do not come into contact with endothelial cells. In arteries and veins the parasite infected cells may cytoadhere to uninfected cells to form rosettes, which has been well documented in literature especially in post capillary venules in autopsy tissue[28–30]. Recently, rolling and adherence have also been observed in graft arterioles[19]. The slowing down of uninfected cells could then provide a mechanism to how these rosettes are formed in the fast flow rates of the arteries and veins.

Thus, we postulate that *Plasmodium*-infected red cells roll or slow down *in vivo* because of the torques generated on the cells due to the combination of the following factors: (i) fluid flow forces; (ii) membrane rigidity of red cells; (iii) structural anisotropy of PRBCs, especially at the trophozoite substage; (iv) collisions of cells; and finally (v) presence of protein-protein interactions between PRBCs and host cells.

These different kinds of anisotropies that arise during parasite infections are either direct or indirect manifestation of opening of various ion channels. It has been shown previously that these ion channels that open up during parasite infection also open up during old age of erythrocytes or under various stress conditions to push the erythrocytes into an apoptotic like process[31,32]. It is also shown that these channels lead to the apoptotic like death of the infected cell. We believe that the molecules that are responsible for opening up these channels in the infected cells may also leak through and open up channels in the neighbouring cells and lead them into the apoptotic pathways. This may explain the possible change in shape and increase of membrane rigidity during infection and hence the increase in the rolling flux of the infected cells and also gives us insights into anaemia caused by the malarial parasite.

Conclusion

From the above study we see that the parasite *P. falciparum* mediates both structural and ionic changes in the infected and its neighboring uninfected erythrocytes. These changes result in changes in the behaviour of these erythrocytes under optical and fluid forces. Thus, this study has great implications in different facets of parasite biology such as cytoadherence, invasion, and immune evasion among others.

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Received: 24 March 2006 *Accepted:* 10 October 2006