Plasmodium-infected red blood cells exhibit enhanced rolling independent of host cells and alter flow of uninfected red cells

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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 synchronized 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 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.

Keywords: Fluid forces, malaria parasite rolling, *Plasmodium*, red cells.

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. It is postulated[1] that the pathogenicity of *P. falciparum* results from its ability to adhere to endothelial cells (cytoadherence) and to erythrocytes (rosetting). A *Plasmodium*-infected red blood cell (PRBC) is said to slow down by rolling, and then undergo adhesion to the venular endothelium[1]. The mechanisms involved are considered to be analogous to the tethering and rolling of leukocytes on vascular surfaces at inflammation sites, for which a large body of information has been collected in the last decade[2,3].

Although considerable work has been carried out to characterize cell adhesion mechanisms, there remains an acute paucity of information on the mechanical events that cause a cell to slow down by rolling in the vasculature. Previous work on cytoadherence of malaria-infected cells has led to the suggestion that rolling as well as final adhesion occurs due to protein–protein interactions between the endothelial cells and the PRBC[4–11]. Assays for rolling have mainly been based on slowing down of cells, and such rolling has been reported to depend on CD36, ICAM1 and P-selectin[12]. Recently, direct visualization has been achieved of adherent or slow-moving infected erythrocytes in graft human microvasculature[13]. Subsequently, such assays have been used to determine involvement of certain signalling pathways of the endothelial cells in the rolling and adhesion phenomenon[14].

We have recently assessed some dynamical properties of parasite-infected as well as normal red blood cells (NRBCs) using an optical laser trap[15]. In such a trap, a tightly focused, linearly polarized laser beam was used to generate optical forces that acted on red blood cells within the focal volume. The magnitude of the optical forces was comparable to capillary fluid forces. It was observed that the morphology of trapped cells, both PRBCs as well as NRBCs, underwent changes upon trapping in the optical field. The electric field gradient of the optical radiation exerted forces that transformed the normal biconcave disk shape of the cell into a folded, cigar-like shape. Furthermore, folded PRBCs subsequently rotated in the laser force field, with the laser focus spot as the pivot, whereas NRBCs did not[16]. Analysis of such behaviour led us to hypothesize that the interaction of the optical field in our trap with the structural (ionic) anisotropy of PRBCs as well as its altered membrane rigidity, gave rise to a polarizability-dependent torque that resulted in cell rotation. Work on the rheological properties of malaria-infected red cells has established that the trophozoite stage cells are least deformable[16], and

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such decrease in deformability has been substantiated in several subsequent reports[17,18]. Due to the focused laser light used in our optical trap, the optical force in these experiments acts on only a small part of the red cell body. How would a force of similar magnitude, acting uniformly over the entire cell body, affect the motion of red cells? Would the fluid forces that are present in the vascular system affect the anisotropic rigid PRBCs in such manner that they acquire a higher propensity to roll? If so, then PRBCs might roll even in the absence of endothelial cells, and inherently possess the ability to slow down and interact with other host cells. To address these questions, we have conducted experiments using a parallel-plate flow-cell and we present here results that yield some new insights into the rolling and slowing down properties of malarial blood samples (IRBCs), a population containing both PRBCs and uninfected red blood cells (URBCs). We show that under hydrodynamic fluid forces, of magnitude comparable to those that act on red cells in the human microvasculature, PRBCs roll and slow down independent of endothelial cells. We also observe that uninfected red cells, present in a culture containing PRBCs, also slow down. This initial slowing down of blood cells appears to depend mainly on the structural anisotropy and altered membrane rigidity of the cells.

Materials and methods

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°C in human erythrocytes of serological type O+ as described earlier[19]. To obtain synchronized cultures, sorbitol treatment according to the method described earlier[20] 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 parasitemia, and diluted 1 : 10 in incomplete RPMI. About 100 μl of this dilution was injected intraperitoneally into naï ve BALB/c mice for the expansion of parasites. Parasitemia 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 centrifuged 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 for use. Nomenclature used for different populations of red cells is as follows: PRBCs, *Plasmodium*-infected red blood cells; URBCs, Uninfected red blood cells exposed to PRBCs in a parasitized blood sample; IRBCs, Red cells of a parasitized blood sample, a population containing both PRBCs and URBCs; 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 was taken through identical treatments of incubation in a humidified chamber containing 5% CO_2 at 37°C. In case of *P. berghei*-containing samples, NRBCs were obtained from uninfected and IRBCs from infected mice.

Flow-cell

The flow-cell was constructed using a glass slide of dimensions 75 mm × 25 mm × 1 mm and a cover slip of dimensions 60 mm × 22 mm × 0.17 mm, with 0.4 mm thick spacers in between to form the flow channel. The effective inner dimensions of the flow channel were 35 mm × 10 mm × 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. 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.

Assessment of rolling cells

The flow-cell was mounted on an inverted microscope (Nikon TE2000-S) and imaged through a 100X oil immersion objective using a CCD camera interfaced to a laboratory computer. Real-time videos of flowing cells were monitored frame by frame, and their velocities 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/s were counted as rolling cells. For each point of measurement, the speed of at least 100 cells was measured.

Results

Rolling of red cells in the absence of endothelial cells

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. We utilized real-time video images to count the number of cells that flowed smoothly versus those which rolled. The sampling volume was determined by the field of view afforded by our optical system. Typically, our depth

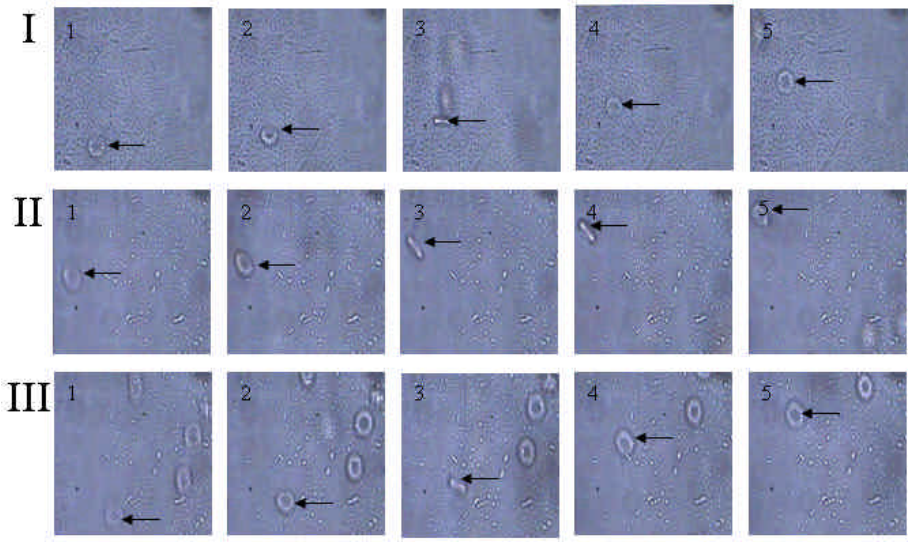

Figure 1. IRBCs were passed through a glass parallel-plate flow cell and video-imaged in real time using an oil-immersed, large numerical aperture 100X objective. Each of the horizontal rows (marked I, II, and III) comprises five temporally consecutive frames that show rolling cells. The time interval between two consecutive frames is 40 ms. Arrows mark the instantaneous position of the rolling cell in each panel.

of focus was up to 50 μm. Taking the flow cell cross-sectional area, red cell concentration and flow rate into account, we compute that up to 1% of all flowing cells were slow enough (< 600 μm/s) to be monitored in our optical system; the remaining 99% red cells was flowing faster than 600 μm/s. All the results discussed below pertain to this 1% of cells observed, which we believe to be representative of the population.

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. Figure 1 depicts a series of video frames that are typical images of three such cells. The fraction of cells in which the camera could trap such cartwheel rolling was small (2% of cells that could be scored). 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.

Percentage of rolling NRBCs and IRBCs

Since actual rolling could be recorded for only a small fraction of cells (about 2% for IRBC and 0.5% for NRBC), the slowing down of cells as they traversed the flow-cell was used as the criterion for rolling. Under the flow condition of 1.4 ml/min, we could observe cells rolling at a velocity of < 400 μm/s. Therefore, cells moving at < 400 μm/s were counted as rolling cells. Similar criterion has been used in earlier reports[12,13]. Only those cells that moved slower than 600 μm/s were counted, and a minimum of 100 cells per assay point were analysed. The distribution of speeds for a population of NRBCs and IRBCs at different stages post-synchronization is shown in Figure 2 *a*. All measurements pertain to a temperature of 25°C. 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-synchronization). The percentage of rolling cells was computed using the cut-off criterion of a velocity of < 400 μm/s. Using this criterion, about 30% of the NRBCs were found to roll, and this value increased to about 50% for the 18–24 h post-synchronized IRBCs (trophozoite stage; Figure 2 *b*). 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 (Figure 3). These results demonstrate that the fraction of slower cells is significantly higher for IRBCs compared to NRBCs.

Factors affecting speed of cells in NRBC and IRBC populations

Dramatic differences in the speeds of red cells were observed in our flow-cell, although the total number of PRBCs was < 3% in the IRBC population (Figure 2). This indicated that the uninfected red cells present in the parasitized blood were also affected, and they might have acquired structural alterations that resulted in a slower velocity. In order to assess such modifications, the effect of the conditioned culture medium (CM) on NRBCs was investigated (Figure 4). As can be observed from Figure 4, the proportion of slower cells increased significantly in the presence of CM, but this effect was not heat-sensitive (Figure 4). To study the behaviour of uninfected red cells in an IRBC population, percoll gradient centrifugation was used to

separate URBCs from PRBCs. Since percoll itself may cause some membrane perturbations, NRBCs were also subjected to percoll treatment for comparison (Figure 4, compare bar 1 and bar 4). It was observed that the URBCs isolated from an IRBC population contained a significantly larger number of slower cells compared to NRBCs subjected to similar percoll treatment (Figure 4). When these sets of URBCs were incubated with complete RPMI medium at 37°C for 12 h, the perturbation on the uninfected red cells seemed to be removed, and the cells reverted to NRBC dynamics (Figure 4). Perturbation caused by the percoll, however, appeared to linger on, and these cells continued to possess a larger number of slower flowing cells compared to red cells that were not subjected to percoll treatment. These results indicate that URBCs exposed to PRBCs are altered through some heat-resistant molecule(s) in the medium, but such alterations were removed in about 12 h of culturing. The trophozoite-enriched red cells showed a significantly higher number of slower cells (Figure 4, bar 8).

Temperature and shear pressure dependence

In order to test whether the fraction of rolling cells depended on membrane rigidity of the red cells, we made a series of measurements on the velocity of NRBCs at different temperatures at the same fluid velocity (Figure 5). The fraction of slow-moving red cells was observed to increase from 35 to 55% as the temperature decreased from 35 to 20°C (Figure 5). The shape and size of red cells did not alter with tempera- ture. Viscosity of the medium also remained unchanged, at 1.13 cPoise at 20 and 35°C.

The effect of changing the shear pressure/force on the percentage of rolling cells was also measured. Reynold's

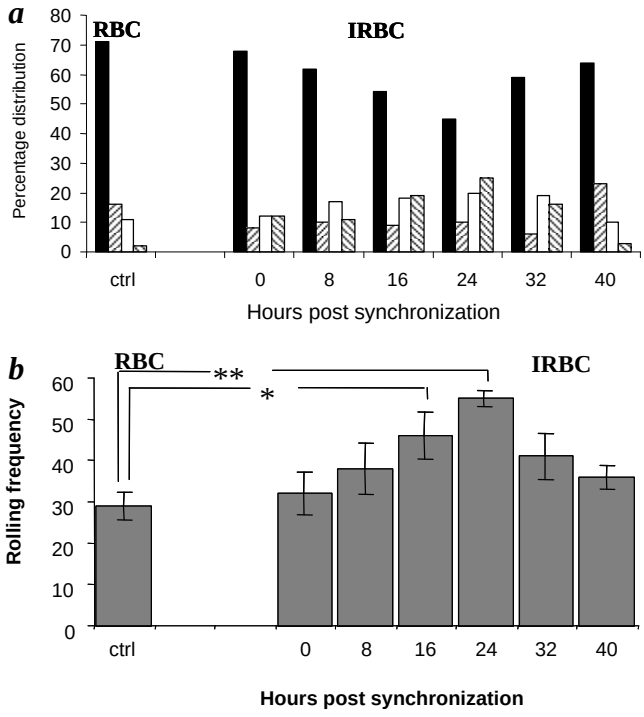

Figure 2. ***a***, Velocity distribution of a population of normal blood cells (RBC) and synchronized *Plasmodium falciparum*-infected RBCs (IRBC; parasitaemia 2.8%) as counted through video imaging. IRBC cells were counted at 8 h time intervals after synchronization. ■ 400–600 μm/s; ▩ 300–400 μm/s; □ 240–300 μm/s; ▨ < 240 μm/s. ***b***, Flux of rolling RBCs and IRBCs. Data from (***a***) were plotted with the criterion of a velocity of < 400 μm/s as a rolling cell. Results are shown as mean ± SEM of four sets of points for each sample. **P* < 0.05 and ***P* < 0.01.

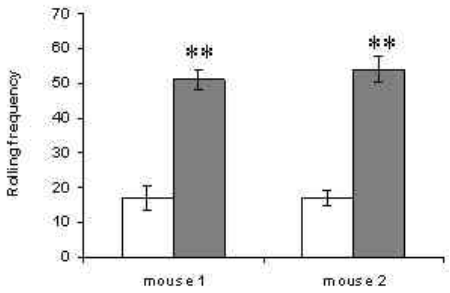

Figure 3. Flux of rolling red cells in an asynchronous culture of *P. berghei*-infected and normal red cells. RBCs were processed after collection from *P. berghei*-infected (~50% parasitaemia) and uninfected BALB/c mice, diluted in complete RPMI media and passed through the parallel plate chamber. Percentage of rolling cells was counted using real-time video images. □ NRBCs from uninfected mouse; ▩ IRBCs from infected mouse. Results are shown as mean ± SEM of four sets of points for each sample. ***P* < 0.01.

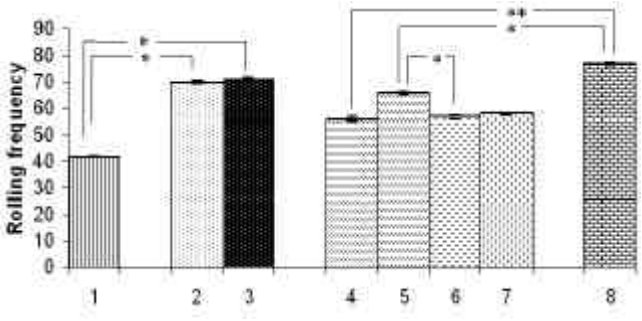

Figure 4. Number of rolling NRBCs and IRBCs obtained after different treatments. NRBCs as well as synchronized IRBCs were incubated in a CO_2 incubator at 37°C for 24 h. The cultures were then centrifuged and the medium obtained from IRBC culture (2.3% parasitaemia) was labelled as conditioned medium. The NRBC pellet was then resuspended in complete RPMI (bar 1); conditioned medium (bar 2); heat-inactivated (10 min at 95°C) conditioned medium at 5% hematocrit and incubated for 12 h in a CO_2 incubator at 37°C (bar 3). Red cells were then harvested, washed and diluted in complete RPMI medium and passed through the flow cell. Another set of IRBC and NRBC cell pellets was resuspended in complete RPMI and passed through a percoll gradient[16]. The trophozoite enriched cell-layer and uninfected red cell layer from IRBCs, and the corresponding uninfected red cell layer from NRBCs were collected, pelleted and washed in complete RPMI medium. One aliquot each from the percoll-separated uninfected red cells from NRBCs (bar 4) and IRBCs (bar 5) was scored immediately for rolling, while another set of aliquots was scored after incubation in a CO_2 incubator at 37°C for 12 h (bar 6: NRBC; bar 7: IRBC). Bar 8 represents enriched trophozoites (10%) after percoll separation of IRBCs. Results are shown as mean ± SEM of four sets from each sample. **P* < 0.05 and ***P* < 0.01.

number deduced for typical operating conditions in our experiments ($< 10^{-1}$) was low enough to ensure that all our measurements conformed to laminar-flow conditions. The wall shear pressure for our flow cell, with a flow rate of 1.4 ml/min and a cross-sectional area of 0.04 cm^2, works out to 0.9 dyn/cm^2 (0.09 Pa). The wall shear pressure was altered by decreasing the flow rate to 50 and 75% of the original value, and measuring the effect on the distribution of cells moving at different velocities. Since the fluid velocity was lower, the frame counts were normalized for these settings. We found that there was no appreciable change in the fraction of rolling cells under these different conditions.

Discussion

Our data indicate that under flow conditions that are comparable to those present in the human microvasculature, some red cells exhibit rolling even in the absence of any host cells or proteins. The fraction of rolling or slower cells is significantly higher in IRBCs compared to NRBCs. We postulate that structural properties and membrane rigidity of infected red cells are mainly responsible for this slowing down. We also observe that a greater fraction of uninfected red cells, present in an IRBC population, flows slower than NRBCs.

The tethering and rolling of flowing leukocytes at the site of inflammation is a well-established phenomenon. Cell–cell interactions slow down the leukocytes, resulting in a rolling motion that finally culminates in the adherence of leukocytes to the signalling endothelial cells[2,3]. The same analogy has been extended towards malaria parasite-infected red blood cells[1,5–13]. Our recent studies of single red cells under optical laser forces (of typical magnitude ~ 10 pN) indicated that red cells rotate based on certain intrinsic structural properties of the trapped cells[15]. Under the action of optical forces, both NRBCs and PRBCs were observed to fold into a semi-cylindrical conformation, but the folded PRBCs started in-plane rotations at speeds up to a few hundred revolutions per minute. A mathematical model, based on the premise that folding of the red cells is due to buckling instabilities, has been developed[21] and predictions made by this model have been experimentally verified. The rotation is largely attributed to the structural anisotropy of the PRBC and occurs in the presence of the optical force field[15]. Based on such observations, we predicted that the parasite-infected anisotropic red cell, may also exhibit inherent rotation under fluid force, and unlike the optical trap which does not allow translational motion, a combination of rotational and translational motion would give rise to rolling cells. When cells flow in blood vessels that are many times larger than the cell diameter, it may be assumed that the flow is laminar, and that cells are subjected to a uniform fluid force field in the direction of the flow. Using our parallel plate flow-cell, whose cross-sectional dimensions (10,000 μm × 400 μm) were also several times larger than the red cells, we observed that most NRBCs align and flow along with the fluid. However, a large proportion of IRBCs appeared to be unable to align and underwent rolling motion. In particular, our measurements indicate that IRBCs at the trophozoite stage possess a higher propensity to roll (and thus slow down) under the normal fluid forces in the capillaries, and do so even in the absence of host proteins.

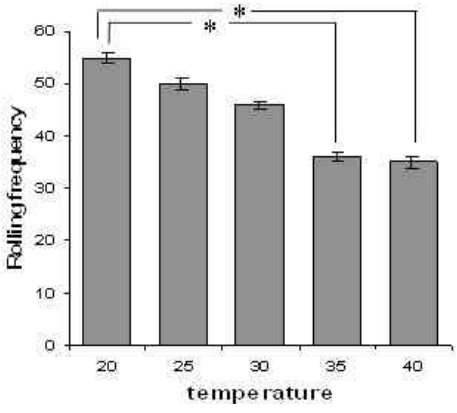

Figure 5. Percentage of rolling NRBCs as a function of temperature. Cells were diluted and maintained at different temperatures using a water bath. Each sample was passed through the parallel plate flow chamber and readings were taken in replicates of four. Results are shown as mean ± SEM of four sets from each sample. *$P < 0.05$.

It has been demonstrated[12] that the rolling of IRBC 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[12]. Under our observation conditions, we observed rolling at shear stress values much smaller than those reported earlier[12,13]. This discrepancy is partly due to the definition of rolling. In earlier reports, cells with flow speeds of < 150 μm/s were counted as rolling, whereas in our studies we counted a cell with a flow speed of < 400 μm/s as a rolling cell. Using a 100X objective and fast video camera, we have a distinct advantage of being able to observe rolling of red cells at a velocity as high as 400 μm/s. Even using the previously-employed criterion[12] of cells moving at < 150 μm/s, our data show that about 25% of IRBCs roll at the trophozoite stage, as opposed to ~ 2% of RBCs (Figure 2 *a*). The shear pressure in our flow cell was 0.9 dyn/cm^2 (0.09 Pa), and cells were found to roll at this low shear pressure. Up to a fourfold decrease in shear pressure did not change the rolling fraction significantly, indicating that rather than shear pressure, membrane rigidity and cell shape are possibly the overriding parameters under these conditions.

For adherence measurements the wall shear stress would be a relevant factor, but for rolling, the force experienced

by a red cell would, perhaps, be a more critical parameter. We compute the value of the fluid force, F, experienced by each flowing red cell as $F = 6\pi\eta av$, where η denotes the viscosity of the medium, a is the radius of the red cell, and v is the flow velocity that is directly measured by video imaging. For simplicity of analysis, we assume the red cell to be a spherical particle. To appreciate the magnitude of forces involved, consider that for human veins with flow velocities ranging from 5 to 20×10^4 μm/s, the values of force experienced by a red cell vary from 20 to 85 nN (the value of viscosity is assumed to be 3.2 cpoise; Figure 6). Similarly in the arteries, where the flow velocity is in the range of $5–40 \times 10^4$ μm/s, the average value of force is calculated to be in the range 20 to 170 nN. In the arteries, however, the flow is in pulses and cells are thus subjected to a fluctuating range of fluid forces. In the capillaries, with an average flow velocity of 100–500 μm/s, the force values are computed to be 40–210 pN (Figure 6). In our flow-cell with a cross-sectional area of 0.04 cm^2 and a flow rate of 1.4 ml/min, fluid velocity across the flow cell computes to be 5800 μm/s. Assuming a red cell flows at a maximum velocity (that of the fluid), the maximum fluid force experienced by it in the parallel-plate flow-cell would be about 750 pN. Therefore, our parallel-plate flow-cell conditions are comparable to those observed in the capillaries/pre-capillaries. The pressure exerted on each red cell is computed as the force per unit area of the red cell (Figure 6).

Several parameters contribute towards rolling and slowing down of the red cells. The erythrocytic developmental stage of the PRBCs is a major determinant. There appears to be a continued increase in the percentage of rolling cells, with maximum fraction observed at 24 h post-synchronization (trophozoite stage). Thus, it appears that the inherent structural anisotropies of trophozoites slow down the PRBCs, providing greater opportunities for them to interact with host endothelial cells, other red cells and platelets. The host endothelial cells also play definitive roles in further rolling and adhesion, since in a majority of 16-h-old trophozoites are already found to be adherent the presence of endothelial cells *in vitro*[1].

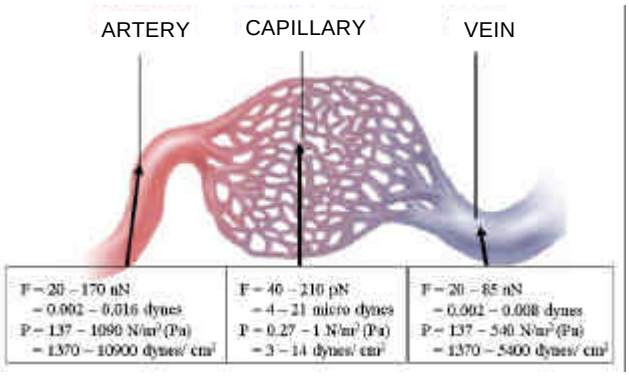

Figure 6. Schematic diagram depicting physiological flow of blood, forces and shear pressure experienced by each red blood cell in different parts of the human vasculature.

Elasticity of the red cell membrane may also be an important parameter for red cell rolling. Red cell elasticity is finely modulated by temperature with an inverse correlation[22]. Since our measurements showed that the percentage of rolling cells increased as temperature was lowered, without concomitant increase in viscosity or any change in cell shape or size, it may be concluded that an increase in membrane rigidity of the red cells led to the enhancement of rolling cells. Microfluidics experiments conducted on single cells in capillaries have shown that normal RBCs, and cells in early stages of malarial infection, pass through channel constrictions of sizes in the range 2–8 μm, with little difficulty. In contrast, increasing stage of infection resulted in considerable blockage, indicating enhanced rigidity[23]. Earlier work had also provided evidence for enhancement of rigidity with infection stage[24], wherein for a system of infected RBCs flowing through different constrictions, the shape recovery time was significantly longer for ring-stage RBCs than for uninfected cells. The elastic response of a healthy RBC to an applied force is likely to be determined essentially by the cell membrane as the inner fluid has no elasticity by virtue of it being purely viscous. In terms of two-dimensional laws of elasticity[25], the elastic shear modulus μ, of the cell membrane and the area compressibility b, determine the dynamics responsible for morphological changes in the RBCs that may be induced by application of fluid forces. b is inversely proportional to the bulk modulus K; this is an experimentally significant parameter in that it relates the change in volume of a solid substance as the pressure on it is altered: $K = \eta(\partial P/\partial \eta)$, where η denotes viscosity and P is the pressure. The elastic properties of the cytoskeleton determine the magnitude of μ, while K is controlled by the almost incompressible phospholipidic bilayer. For healthy RBCs, it is experimentally established[26] that $K \gg \mu$. Thus, the rolling that is observed is likely to proceed in such manner that the elastic properties of the cytoskeleton come into play while the overall area of the cytoskeleton remains constant. Recent work has confirmed that the deformability of malaria-infected RBCs is considerably reduced[17,18]. This reduced deformability property will have to be incorporated in any physical model of the rheological properties of flowing RBCs, along with parameters such as elastic properties of the cytoskeleton. In this connection it is also of interest to note a recent cogent review of how the malaria parasite affects the permeability of the RBC membrane[27].

In the case of uncomplicated malaria patients, the per cent of parasitized cells is generally around 0.1–1 (4000–40,000/μl), although in severe malaria cases[28] this may go up to > 20%. Thus the IRBC samples used in our study (parasitemia < 3%) were in the physiological range expected in uncomplicated malaria patients. Our observation that the large number of uninfected red cells present in a parasitized culture, had a higher fraction of rolling cells even at these low parasitemia values, has serious physiological implica-

tions. Red cell deformability measured with ektacytometry was found to be reduced in uninfected URBCs of malaria patients along with PRBCs[29]. Exo-antigens of *Plasmodium*, which increased the membrane rigidity of neighbouring uninfected URBCs, have been reported earlier and these factors were found to be heat-sensitive[30]. However, Paulitschke and Nash[31] could not subsequently confirm the presence of exo-antigens. Our observations indicate that such factors are heat-resistant and are unlikely to be exo-antigens. The perturbations were transient since the exposed cells were observed to revert to the behaviour of NRBCs within 12 h. Further analysis using the spent culture medium may reveal better the nature of the molecular players that cause URBC alterations. Such alterations in uninfected red cells may help in the slowing down of URBCs, favouring URBC–IRBC interactions. In larger arteries and veins, where the bulk of the flowing cells may not come in contact with the endothelial cells for cytoadhesion, the slower/rolling PRBCs and URBCs would have enhanced chances of interaction. Such interactions would result in the formation of rosettes, which have been implicated in the pathophysiology of *P. falciparum*[32–34]. Cytoadhesion has been mainly documented in the capillary and post-capillary venules using autopsy tissues[18]. Recently, however, the use of graft human microvasculature has demonstrated that rolling and adhesion are observed in the arterioles as well[13].

It is apparent that membrane rigidity is not the only parameter of importance in the rolling of cells, since mature schizont stages have membrane rigidity comparable to trophozoite stages[31], and yet the fraction of rolling cells was found to be lower for the schizont stages. It is our postulate that the anisotropic shape and structure of the trophozoites play an important role as well. Unlike near-spherical leukocytes (and schizonts), trophozoites are non-spherical, and exhibit larger anisotropy compared to healthy red cells. Under normal circumstances, the NRBCs are able to fold and flow with minimum drag resistance. However, *Plasmodium* parasite not only makes the cell more rigid, but also distorts its shape. Structural anisotropy is also inherent in the early ring and trophozoite-infected IRBCs, since the parasite is physically located on one side of the biconcave red cell. The imaging of intracellular distribution of free Ca^{2+} ions during development of the malaria parasite shows marked spatial anisotropy[35]. With an asymmetric rigid red cell, the behaviour of IRBCs in the flowing vasculature under fluid force perhaps parallels that of a whirling flower falling under gravitational force. However, in the schizont stage, erythrocytes are less anisotropic and nearly spherical, and would flow smoother in a laminar flow. Thus, although the membrane rigidity of schizonts is comparable to that of trophozoites[31], the propensity to roll is less for the former. A similar trend was observed in the single-cell assays in our optical trap experiments, and the rotation velocity was found to be highest for trophozoites[15].

Apart from membrane rigidity, cell deformability, cell geometry and anisotropy, cell–cell collisions are also likely to play a role in the rolling of red cells. It has been demonstrated that the presence of RBCs enhances the rolling of leukocytes[36]. Under physiological conditions (40–50% hematocrit), red cell collisions are also likely to play a role. Thus, we postulate that *Plasmodium*-infected red cells roll *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. Studies on the interactions of infected erythrocytes with uninfected RBCs and endothelial cells are relevant in view of the pathophysiology observed in cases of severe malaria. Microfluidic devices and optical laser traps that can observe the behaviour of single cells under certain force fields will also allow us to understand better the behaviour of IRBCs in the microvasculature.

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