Abnormalities in the Mechanical Properties of Red Blood Cells Caused by Plasmodium Falciparum


Although changes in the mechanical properties of infected red cells may contribute to the pathophysiology of malaria, such changes have not previously been described in detail. In this study, the physical properties of individual cells from both clinical and cultured samples infected with *Plasmodium falciparum* were tested using micropipette aspiration techniques. Cells containing ring forms took about 50% longer to enter 3 μm pipettes compared with unparasitized cells, and there was a similar increase in the critical pressure required to induce cell entry. These abnormalities were similar in clinical and cultured samples. More mature cultured parasites (i.e., trophozoites and schizonts containing pigment) caused much greater loss of deformability, with entry time and pressure increased four to sixfold. The decrease in deformability of the ring forms was attributable to a deficit in cell surface area/volume ratio (based on micropipette measurement of the surface area and volume of individual cells) and slight stiffening of the cell membrane (shear elastic modulus increased 13%, as measured by pipette aspiration of small membrane tongues). Measurement of the rate of cell shape recovery indicated that the membrane of parasitized cells was not more viscous. The main factor in the drastic loss of deformability of the trophozoites and schizonts was the presence of the large very resistant parasite itself. Otherwise, the cell surface area/volume deficit was slightly less and membrane rigidification slightly greater compared with ring forms. The above abnormalities should cause the trophozoites and schizonts to have great difficulty in traversing splenic or marrow sinuses and could contribute to microvascular occlusion and sequestration. On the other hand, the ring forms may be expected to circulate relatively unhindered.

*Plasmodium falciparum* causes the most virulent form of malaria in humans. It differs from other plasmodial species in that only the less mature intraerythrocytic “ring” forms are found in the circulating blood, while the more mature pigmented forms (trophozoites and schizonts) are sequestered in the microcirculation of the internal organs. Infected patients suffer from ischemic complications, in particular cerebral malaria, which is a major cause of mortality. The major factor in parasite sequestration and microvascular occlusion is currently thought to be the tendency for the infected cells to adhere to vascular endothelium. However, changes in the cellular mechanical properties may also contribute to the pathology. Abnormalities in flow behaviour could add to the tendency to plug narrow vessels and cause increased resistance to perfusion. Trapping of parasitized cells in the reticuloendothelial system might be promoted by mechanical changes, adding to the cell destruction and anemia associated with malaria.

Surprisingly little is known about the changes in cellular mechanics caused by malarial infestation. Miller et al. demonstrated that infected red cells from monkeys with *P coatneyi* and *P knowlesi* had impaired flow rates through micropore filters, particularly if mature parasites were found in the blood. A report of impaired filterability of blood from humans with *P falciparum* was made by Lee et al., but their control data was scanty and the red cells were not separated from the other components of the blood. Cranston et al. made a more detailed study of individual cells subject to shear forces in a transparent, rotating cone-plate device (Rheoscope). They found that cells containing cultured ring forms of *P falciparum* elongated less, and also showed a tendency to tumble rather than become stably aligned with the shear flow. These cells recovered their shape more slowly than unparasitized cells after cessation of shearing. Mature parasites caused nearly all cells to tumble rather than deform. A deficit in cell deformability was thus demonstrated, but the structural basis was not clear. Nor can the results be easily extrapolated to cell behaviour in the microcirculation and reticuloendothelial system, where cells must squeeze through narrow capillaries or slits.

In general, red cell deformability is dependent on the size and shape of the cells (particularly the surface area to volume ratio), the viscoelasticity of the cell membrane and the state of the cytoplasm (usually the cytoplasmic viscosity, but in malaria the presence of the parasite itself may affect deformation). In this study we have used micropipette aspiration techniques to obtain information on the deformability of individual cells and on each of the contributing factors. Deformability was assessed by measuring the time and the pressure required for complete entry into pipettes with internal diameter 3.0 to 3.5 μm. Cell surface area and volume were determined by partially aspirating cells into 2 μm pipettes. Membrane viscoelasticity was tested by measuring the membrane shear elastic modulus and the time constant for extensional recovery. The product of the elastic modulus and the time constant represents the membrane viscosity. Parasitized cells were obtained from newly diagnosed, untreated patients and from cultures of *P falciparum*. To our knowledge, this study represents the first detailed description of the rheological changes induced by parasite...
entry and maturation in the human red cell, and the first comparison of cultured and wild types.

METHODS

Blood Samples

Venous blood was drawn from eight patients newly diagnosed as suffering from falciparum malaria, judged by identification of parasites using Giemsa stained blood films. Sampling was carried out with informed consent and before commencement of treatment. For the first three patients, the blood was anticogulated with 1.5 mg/mL K$_2$EDTA and measurements were started within two hours. For the next five patients, blood was drawn into citrate dextrose solution and stored overnight in the cold before measurements were made. Parasitemia varied between approximately 1% and 13%. Unless otherwise stated, for micropipette analysis the blood was diluted a thousand-fold in phosphate buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na$_2$HPO$_4$, 0.2 g/L KH$_2$PO$_4$, 1 g/L glucose, pH 7.4) and 2% autologous plasma.

Samples were also obtained from six separate cultures of *P. falciparum*,!*13* The cells were suspended at about 5% haematocrit in culture medium (RPMI 1640) plus 15% AB serum and cultured as described by Trager and Jensen.!*14* Parasitemia averaged 9%. Unless otherwise stated, for micropipette analysis the culture was diluted 100-fold in a mixture of PBS and culture medium (6.5:1) with a final serum concentration of 2%. In some cases the cultures were synchronised by 5% D-sorbitol,*!*15* to allow parasites to be studied at a particular stage of maturation.

Micropipette Analysis of Cell Mechanics

The microscope/video and micropipette system was similar to that previously described.!*16* The dilute cell suspension was placed in a chamber made of a glass slide and coverslip separated by a U-shaped gasket. Two percent plasma or serum was included in the medium (see above) to minimize cell-pipette adhesion. The chamber was placed directly onto the microscope stage (for measurements at room temperature of about 21°C) or in a slot in a heated metal block so that the chamber temperature was held at 37°C. A micropipette was connected to a hydrostatic pressure system with resolution of 0.05 mmH$_2$O, and manipulated to enter the open side of the chamber. The tip of the pipette and surrounding cells were viewed by black and white video camera and monitor, and images were recorded on a videotape recorder capable of consecutive, single frame playback. The images were analyzed by mixing the video recording with the video output of a microcomputer. Cell dimensions on the monitor screen were measured by using a Basic program to generate moveable markers on the screen. The separation of the markers could be recorded or used directly in calculations. In each experiment, parasitized and nonparasitized cells from the same suspension were compared using the same micropipette; cells containing parasites and control parasite-free cells were measured alternately to avoid any time based bias.

The micropipette methods are illustrated in Fig 1, which shows ring forms under test. The different methods of analysis were as previously described and are only outlined here.

Pipette entry. Pipettes with internal diameter of 3 to 3.5 μm were used to completely aspirate RBC. The critical pressure required for cell entry (Pe) was tested by raising the pressure in small steps (0.05 to 0.1 mmH$_2$O) every few seconds until the cell was fully aspirated. Pe depends strongly on pipette diameter and averaged 0.4 mmH$_2$O for nonparasitized cells in this study. The time for entry (Te) at a fixed pressure (1 to 3 mmH$_2$O depending on pipette diameter) was measured from video recordings and averaged about 0.5 seconds for nonparasitized cells. Pipette entry measurements were carried out at room temperature and at 37°C. Typically ten to 20 parasitized cells and 20 to 40 nonparasitized cells were tested in a given suspension.

Cell surface area and volume. The surface area (SA) and volume (V) of individual cells were measured by aspirating them into approximately 2 μm pipettes at a pressure of 2 cm H$_2$O. The cells were then constrained to assume a cylindrically symmetrical form with a smooth spherical outer portion. By measuring the dimensions of the cells (diameter of spherical portion, length of cylindrical portion) and pipette diameters, SA and V could be calculated. Measurements were carried out at 37°C and typically 20 parasitized cells and 40 controls were measured in a given suspension.

Membrane viscoelasticity. The resistance of the membrane to deformation was characterized by measuring the membrane shear elastic modulus (μ, resistance to shear deformation at constant area) and the time constant for shape recovery after elongation of the cell (τc). The product μτc represents a measure of the surface shear viscosity of the membrane (ηm). Both μ and ηm are mainly determined by the protein structure of the membrane cytoskeleton.!*17* The membrane shear elastic modulus was determined by measuring the length (L) of a membrane tongue aspirated from the dimple region into an approximately 1.5 μm diameter pipette (Fig 1d). L was measured at 3 to 5 pressures (P) in the range 1 to 4 mmH$_2$O, and the elastic modulus μ calculated from dL/dP as previously described.*9*

To measure the time constant for extensional recovery, cells were first suspended in PBS containing 0.3 g/dL human serum albumin and allowed to settle and attach to the bottom of the micropipette...
chamber. The chamber was then flushed with PBS containing 2% autologous plasma (for clinical samples) or PBS mixed with culture medium 6.5:1 (for cultured samples). Cells that were point attached to the bottom were elongated by pulling them from one side using a micropipette and then released (Fig 1e and f). The rate of their shape recovery was measured from video recordings and analyzed as previously described\(^9\) to obtain the time constant for the essentially exponential recovery process. Measurements were made at room temperature, on an average of six cells of each type in each sample.

RESULTS

Pipette entry experiments were carried out on clinical and cultured samples, at room temperature (21°C) and/or at 37°C. The readily recognizable ring forms (see Fig 1) were tested separately from more mature pigmented forms—the latter included both trophozoites and schizonts, which could not reliably be distinguished from each other. Pooled data from two to six experiments in each category are shown in Figs 2 and 3 for the critical pressure and time required for cell entry, respectively. Values for individual parasitized cells are shown, expressed relative to the median value for control nonparasitized cells in the same suspensions.

For ring form parasites, the pooled data indicate a 50% to 60% elevation in median critical pressure and entry time. In the separate experiments, the ratio of ring form/control median values was 1.6 ± 0.3 for the critical pressure (mean ± SD from eight experiments) and 1.7 ± 0.4 for entry time (mean ± SD from 14 experiments). These ratios tended to be slightly lower at 37°C than at 21°C but this trend was not statistically significant in six comparative experiments. Cells containing more than one ring form (seen more often in cultured than clinical samples) frequently would not enter the pipettes at all; those that did had median entry time increased 110% (11 cells, data not shown).

There is clearly considerable scatter in the data for parasitized cells; the coefficient of variation (c.v.) for the entry parameters averaged 41% for rings compared with 27% for nonparasitized control cells. The mean values for the entry parameters are greatly influenced by the few extremely high values and their ratio to controls is higher than the ratio of medians. The scatter may reflect the presence of rings of different ages in unsynchronized cultures, although a synchronized culture of young rings had a c.v. of 38%.

Maturation of cultured parasites caused a marked further loss of deformability. The pooled data for pigmented parasites (Figs 2 and 3) indicate that critical pressure was increased about fourfold and the entry time sixfold compared with controls, regardless of the measurement temperature. In the separate experiments, the ratio to controls was 4.2 ± 1.3 for the critical pressure (mean ± SD from three experiments) and 6.7 ± 4.1 for entry time (mean ± SD from six experiments). The pigmented parasite itself was spherical, stiff and very slow to deform. Since larger parasites tended to cause greater impairment in deformability, it is likely that the schizonts caused greater changes than the trophozoites. However, this cannot be definitely inferred from our data because each experiment was carried out using a suspension that contained both trophozoites and schizonts (average...
trophozoite parasitaemia 2.4%, schizont parasitaemia 1.7%, judged from counts on Giemsa stained thin films). The variability in the entry data was very high (average c.v. = 65%), and this presumably reflects the mixed nature of the preparations.

Geometrical parameters for parasitized and nonparasitized cells are compared in Fig 4. Neither ring forms nor more mature pigmented parasites caused a significant change in cell surface area. However, in every sample of ring forms tested, whether cultured or of clinical origin, the mean cell volume was higher than control (elevation, 11.3% ± 5.0%; mean ± SD for eight comparisons). Consequently, the surface area to volume ratio was decreased for ring forms (by 8.0% ± 2.2%). Cells containing more than one ring form had a larger deficit in surface area/volume (average decrease, 13.2% in three experiments). Cells with trophozoites or schizonts had a less marked elevation in volume (6.7% ± 7.6%, five comparisons) and thus a lesser decrease in surface area to volume ratio (4.5% ± 4.5%).

Data for membrane viscoelasticity are shown in Fig 5. In eight experiments (three on clinical samples and five on cultured samples) the mean membrane shear elastic modulus was consistently slightly higher for ring forms than for nonparasitized cells (Fig 5A). The difference was not significant in any one experiment, but comparison of the eight pairs of data showed a significant mean elevation of 13% (P < .01 by paired t test). Some cells containing pigmented parasites retained a roughly discoidal shape, with a flat region suitable for analysis of elasticity. These were compared with nonparasitized cells in four experiments and had a membrane elastic modulus elevated 50% on average (P < .05 by paired t test). Overall, the data indicate that parasites cause an initial, slight stiffening of the cell membrane which increases during maturation.

In four experiments (Fig 5B), the time constant for extensional recovery was consistently lower for ring forms than for controls (22% decrease, P < .01 for pooled data compared by Student's t test). The product of μ × τc, representing the membrane surface viscosity, was slightly but not significantly decreased for ring forms. Thus one can state that the ring form cells recovered their shape more quickly because their membrane was stiffened but not more
viscous. The time constant was not measured for more mature parasitized cells as they did not attach well to the chamber surface and tended to detach rather than elongate when pulled from one side. However, it was notable that the membrane of these cells did deform slowly when sucked into a pipette, suggesting that the membrane viscosity had increased or that the cell had become significantly dehydrated.

**DISCUSSION**

This study shows that invasion and maturation of *P. falciparum* causes progressive loss of red cell deformability, assessed by measuring the pressure and time required for cell entry into 3 μm pipettes. An early abnormality was already detectable in a synchronized culture of "young" ring forms. A much more drastic defect was evident when trophozoites or schizonts were present. Such abnormalities could depend on several factors: the viscoelasticity of the membrane (which affects its ability to shear and fold during pipette entry), cell surface area and volume (which determine the degree of deformation required and limit its ultimate extent), and the internal state of the cell, including the properties of the parasite itself and any attachment to the membrane.

During maturation from ring to pigmented stages, the parasites enlarged. The pigmented parasites were rigid and very viscous, and appeared directly to become the major cause of the loss of cell deformability. For the ring forms, a combination of mechanical changes probably caused the defect. The cells had a deficit in the surface area to volume
sites are a good cultured origin. It seems that in this aspect, cultured parasitized cells, so that maturation of the parasite from the ring form to the merozoite). Cells containing mature parasites would probably be unable to pass through these slits. This fits with the finding that the narrow sinuses of patients dying from cerebral malaria are packed with parasitized cells. Because of their lack of deformability, mature parasites may not be able to gain egress from this region and could obstruct the release of red cells from the marrow. Thus changes in cell deformability could contribute to the anemia of malaria both by promoting cell destruction and by blocking release of new cells.

The ischemic complications of falciparum malaria are currently thought to arise mainly from adhesion of cells containing mature parasites to vascular endothelium. The mechanical abnormalities seen here, however, could also cause appreciable microcirculatory obstruction. The more grossly abnormal cells might become trapped or at least cause delay at capillary entrances. Moreover, in vessels lined with adhered cells, the flow in the central region would also be impaired by the presence of any poorly deformable cells. Infection with malarial parasites of other species (such as vivax or malariae) leads to less drastic clinical complications. It is interesting to speculate whether this correlates with the rheological changes induced by these infections and we are currently attempting to investigate this area.

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