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Rosetting

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Definition

Rosetting is the spontaneous binding of *Plasmodium*-infected erythrocytes to uninfected erythrocytes to form clusters of cells.

Introduction

Rosetting is the most well-established *Plasmodium falciparum* virulence factor contributing to life-threatening malaria in humans. Rosettes form during the blood stage of malaria infection, when erythrocytes infected with mature asexual parasites (pigmented trophozoites and schizonts) bind spontaneously to uninfected erythrocytes to form clusters of cells (Fig. 1). The association between rosetting and severe malaria is well-documented, and the potential for rosetting to contribute to the pathophysiology of severe malaria by obstructing microvascular blood flow and modifying uninfected erythrocytes has been demonstrated. However, the molecular mechanisms of rosetting and the biological function(s) of rosetting remain unclear.

P. falciparum Rosetting Is Associated with Severe Malaria in Sub-Saharan Africa

Rosetting is an adhesion phenotype that varies between *P. falciparum* isolates. To study the relationship between rosetting and malaria severity, blood samples have been collected from malaria patients and cultured for 12–24 h to allow the parasites to mature sufficiently to form rosettes. This is required because ring-stage parasites present in patients' blood samples do not form rosettes, and mature stages are concealed in microvascular beds in vivo by a process called sequestration and cannot be sampled directly from the blood. After short in vitro culture, the rosette frequency (percentage of mature infected erythrocytes in rosettes) can be assessed by microscopy. Figure 1 shows a *P. falciparum* culture with a rosette frequency of >80 %. Numerous studies in sub-Saharan Africa have shown that rosetting varies from 0 % to >90 % in isolates collected from malaria patients and that rosetting levels are significantly higher in parasite isolates from severe malaria patients, compared to isolates from patients with uncomplicated disease (summarised in Doumbo et al. 2009). Most studies suggest that rosetting is associated with all clinical forms of severe malaria, including impaired consciousness and coma (cerebral malaria), severe malarial anemia with a hemoglobin level of <5 g/dl, and respiratory distress (difficulty breathing) (Doumbo et al. 2009).

Despite a consistent body of work linking rosetting with severe malaria in moderate- and high-transmission areas in sub-Saharan Africa, studies from low transmission areas such as Southeast

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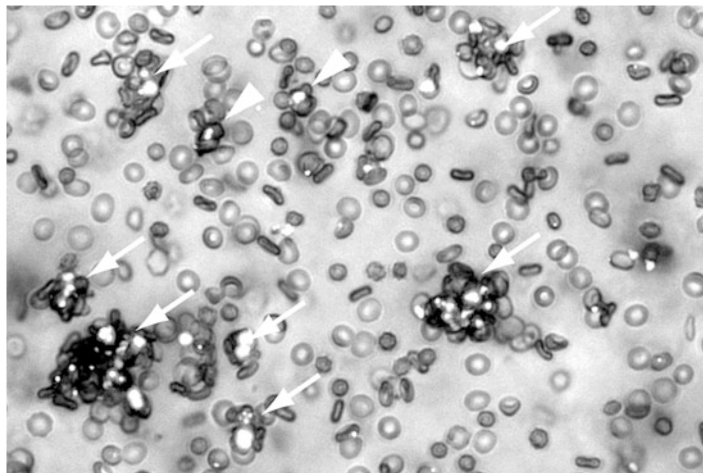

Fig. 1 *P. falciparum* rosetting viewed by combined fluorescence and bright field microscopy. Parasite DNA is stained with ethidium bromide and shows as white patches within infected erythrocytes. Rosettes are clusters of infected and uninfected erythrocytes. Single rosettes (*arrowhead*) are usually seen in static assay conditions, whereas large multi-rosettes comprising aggregates of multiple infected and uninfected erythrocytes (*arrows*) form in vitro when cultures are examined under flow conditions similar to those experienced in vivo

Asia are few and do not show a clear association between rosetting and severe malaria (reviewed in Rowe et al. 2009). Further studies are needed to examine the relationship between rosetting and malaria severity in low transmission areas.

Human Erythrocyte Polymorphisms and Rosetting

The association between parasite rosette frequency and severe malaria supports a role for rosetting in the pathophysiology of severe disease but does not prove a causal link. Further evidence to support a role for rosetting in severe malaria comes from human genetic studies. In malaria-endemic regions, there are common human erythrocyte polymorphisms affecting the receptors for rosetting on uninfected erythrocytes that reduce the ability of *P. falciparum* parasites to form rosettes. These include blood group O (Carlson and Wahlgren 1992) and complement receptor 1 (CR1) deficiency (Rowe et al. 1997), both of which provide significant protection against life-threatening malaria (Cockburn et al. 2004; Rowe et al. 2007). The data for blood group O are consistent across different malaria-exposed populations, although the magnitude of the protective effect varies. However, the effect of CR1 deficiency varies in different populations, possibly related to the intensity of malaria transmission in different areas (Sinha et al. 2009). The protective effect of hemoglobin S and alpha-thalassemia may be partly due to reduced rosetting (Carlson et al. 1994). The fact that specific rosette-reducing polymorphisms occur at high frequency in malaria-exposed populations and protect against severe malaria and death is a strong evidence to support a causal role for rosetting in the pathophysiology of severe malaria.

How Does Rosetting Contribute to Severe Malaria: Parasite Density?

An animal model in which *P. falciparum* parasites infect splenectomized *Saimiri* monkeys has shown that rosetting parasites have higher in vivo multiplication rates and reach higher parasite

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densities than isogenic non-rosetting parasites (Le Scanf et al. 2008). It is unclear whether this is due to increased invasion efficiency or reduced immune clearance of the rosetting parasites compared to the non-rosetting ones. There is also evidence in humans for a significant positive correlation between parasite rosette frequency and parasite density (Rowe et al. 2002a), although this was not found in all studies (Carlson et al. 1990). Detailed statistical analysis has shown that in some studies, parasite density in the peripheral blood is more closely associated with severe disease than rosette frequency, and the significant association between rosetting and severe malaria is lost when parasite density is included in the statistical model (Warimwe et al. 2012). These data could suggest that rosetting contributes to severe malaria pathophysiology by promoting high-density infections in vivo. However, the relationship between parasite density and severe malaria remains poorly understood. High parasite density seems to be necessary but not sufficient to cause severe disease, as many children have high parasite density but do not develop the clinical features of severe malaria (Goncalves et al. 2014).

How Does Rosetting Contribute to Severe Malaria: Microvascular Obstruction?

Experimental evidence suggests that rosetting may contribute to the pathophysiology of severe malaria by causing obstruction to blood flow in small blood vessels. Reduced blood flow as a result of *P. falciparum* infected erythrocyte sequestration in the microvasculature has been documented in ex vivo animal models (Raventos-Suarez et al. 1985) and in living humans (Dondorp et al. 2008). Studies using ex vivo rat microvasculature have shown that rosetting parasites cause significantly greater obstruction to blood flow than isogenic cytoadherent, non-rosetting parasites (Kaul et al. 1991). Microvascular obstruction is thought to be the key fundamental pathological change in severe malaria, leading to hypoxia, acidosis, endothelial dysfunction, and proinflammatory and procoagulant changes, that together can lead to death of the host (Moxon et al. 2011). Rosetting parasites may contribute to sequestration and microvascular obstruction by simultaneously binding both uninfected erythrocytes and endothelial cells (Adams et al. 2014) (Fig. 2, white arrow). Alternatively, because infected erythrocytes can form rosettes with either infected or uninfected cells, the presence of cytoadherent non-rosetting infected erythrocytes lining blood vessels walls may anchor rosetting parasites in the microvasculature as illustrated in Fig. 2 (black arrow). Both of these processes could lead to aggregations of infected and uninfected erythrocytes, as seen in ex vivo animal model studies and human postmortem studies. The orthogonal polarization spectral imaging technique used by Dondorp et al. to observe microvascular obstruction in vivo in human rectal mucosa (Dondorp et al. 2008) is not able to resolve individual erythrocytes clearly; therefore, it is not yet possible to visualize rosetting in living humans.

How Does Rosetting Contribute to Severe Malaria: Erythrocyte Damage?

Rosetting has been specifically associated with severe malarial anemia in some studies (Newbold et al. 1997). However, a mechanism for this has been lacking. Recent work shows that the uninfected erythrocytes in rosettes show membrane modifications that may target them for phagocytic removal (Uyoga et al. 2012). The excessive removal of uninfected erythrocytes is the hallmark of severe malarial anemia, and Uyoga et al. (2012) provide a possible mechanism by which rosetting may contribute to this process.

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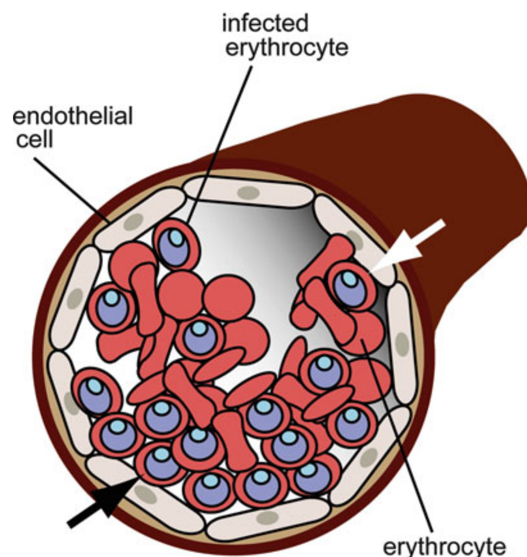

Fig. 2 Diagram to illustrate how *P. falciparum* rosetting may contribute to sequestration and microvascular obstruction. Rosetting infected erythrocytes can either bind directly to endothelial cells (*white arrow*) or can bind non-rosetting infected erythrocytes that are adhering to the vessel endothelium (*black arrow*)

Why Do Parasites Form Rosettes?

If rosetting contributes to host tissue damage and death, why is the phenotype maintained? It is possible that rosetting contributes to parasite fitness and transmission success through its ability to promote high parasite density in vivo as described above. However, the precise way in which rosetting benefits the parasite is not yet clear. Rosetting could enhance invasion efficiency, by targeting invasive merozoites from a rosetting schizont directly into the uninfected erythrocytes forming the rosette. Experimental tests of this hypothesis in vitro have failed to show any effect of rosetting on invasion (Deans and Rowe 2006), and erythrocytes in a rosette are not preferentially targeted for invasion (Clough et al. 1998). The high in vivo multiplication rate of *P. falciparum* rosetting parasites in *Saimiri* monkeys described above could be due to invasion efficiency, but this has not been proven. An alternative explanation is that rosetting is an immune evasion mechanism that helps parasites avoid clearance by either innate or acquired host immune responses. There are as yet no published studies addressing his possibility.

Molecular Mechanisms of *P. falciparum* Rosetting

Parasite-Derived Adhesion Molecules on the Surface of Infected Erythrocytes

Detailed studies of culture-adapted *P. falciparum* parasites selected for rosetting have shown that the parasite molecules responsible for rosetting are members of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, encoded by group A *var* genes (Rowe et al. 1997; Vigan-Womas et al. 2008). Furthermore, field studies have shown a positive correlation between transcription of group A *var* genes and parasite rosette frequency in clinical isolates (Bull et al. 2005). PfEMP1 variants are high-molecular-weight proteins expressed on the surface of infected erythrocytes, which can undergo switching from one asexual blood stage cycle to the next, as part of the process of antigenic variation in malaria parasites. The involvement of a variant surface antigen family in rosetting explains the variation in the phenotype seen in different *P. falciparum* isolates collected

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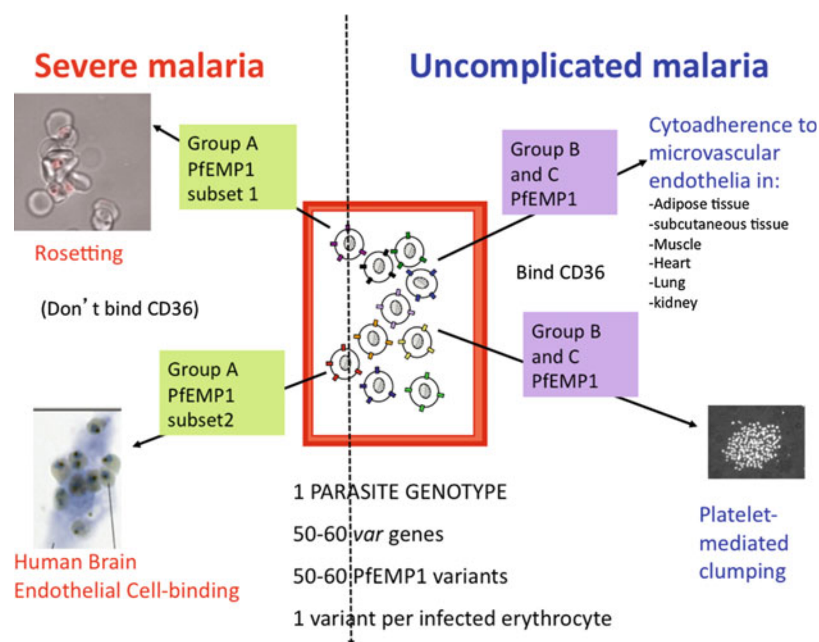

Fig. 3 Diagram to illustrate how particular subsets of PfEMP1 mediate different adhesion phenotypes, which are in turn associated with different clinical forms of malaria. Current evidence suggests that rosetting and binding to human brain endothelial cells (HBEC) are the adhesion phenotypes with the strongest association with severe malaria. Rosetting and HBEC binding are each mediated by different subsets of PfEMP1 encoded by group A *var* genes

from malaria patients. Only a specific subset of the PfEMP1 family is able to mediate rosetting, with other members of the family being responsible for adhesion to other human cell types such as platelets and endothelial cells (Rowe et al. 2009) (Fig. 3).

Existing data suggest that the N-terminal region of rosette-mediating PfEMP1 variants binds to receptors on uninfected erythrocytes to form rosettes (Fig. 4a). Polyclonal and monoclonal antibodies against the N-terminal region are highly effective at disrupting rosettes (Ghumra et al. 2011; Vigan-Womas et al. 2011) (Fig. 4b, c), although antibodies to other domains also have rosette-disrupting effects at higher concentrations (Ghumra et al. 2011).

While it is clear that PfEMP1 plays a major role in *P. falciparum* rosetting, it remains possible that other parasite proteins could act as accessory molecules in adhesion. The RIFIN and STEVOR variant surface antigen families are possible candidates, and recent work suggests a possible role for STEVOR in rosetting in the *P. falciparum* laboratory clone 3D7 (Niang et al. 2014). Further work is needed to determine if STEVOR proteins contribute to rosetting in other *P. falciparum* laboratory strains and clinical isolates.

Uninfected Erythrocyte Receptors

Several different erythrocyte surface molecules have been identified as rosetting receptors, indicating that rosetting is not a simple receptor-ligand interaction between one parasite adhesion molecule and one receptor on infected erythrocytes but is a complex phenotype involving multiple mechanisms (Mercereau-Puijalon et al. 2008) (Fig. 5). The A and B blood group trisaccharides, which are attached to various glycoproteins and glycolipids on the erythrocyte surface, are important receptors for many *P. falciparum* rosetting isolates (Carlson and Wahlgren 1992). Rosetting parasites form larger, stronger rosettes when grown in group A or B blood compared to group O blood, with the preference for A or B varying between isolates. However, group O erythrocytes (which lack one sugar molecule present in A [N-acetyl-galactosamine] or B [galactose] erythrocytes) and "Bombay"

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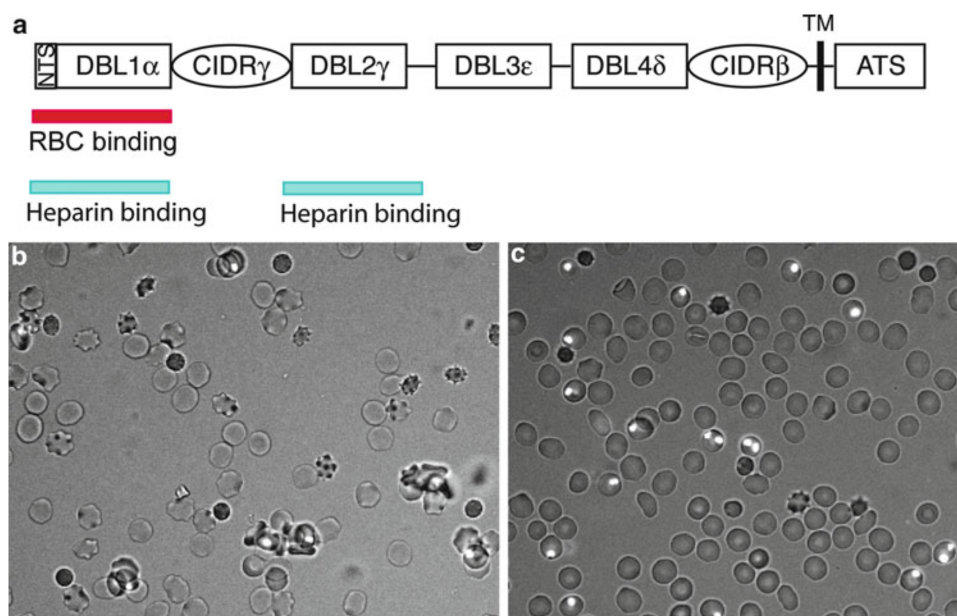

Fig. 4 Antibodies to PfEMP1 at low concentrations abolish rosetting. (**a**) Diagram of a rosette-mediating PfEMP1 variant (IT4var09 expressed by the *P. falciparum* rosetting clone IT4/R29) (Rowe et al. 1997). The protein has one transmembrane domain (TM) and a cytoplasmic acidic terminal segment (ATS). The extracellular region consists of tandemly arranged cysteine-rich domains that are unique to *Plasmodia* called "Duffy binding like" (DBL) or "cysteine-rich interdomain region" (CIDR). The N-terminal segment (NTS) and the first DBL domain together comprise the main erythrocyte binding region that mediates rosetting in IT4/R29 parasites via CR1 and blood group A sugars on uninfected erythrocytes. The NTS-DBL1α domain and the second DBL domain bind sulfated glycoconjugates such as heparin and heparan sulfate (Adams et al. 2014), and these interactions may contribute to rosette formation. (**b**) Rosetting of IT4/R29 parasites in vitro. (**c**) Rosetting is abolished by 10 μg/ml of polyclonal antibody to NTS-DBL1α (rabbit total IgG preparation) (Ghumra et al. 2011)

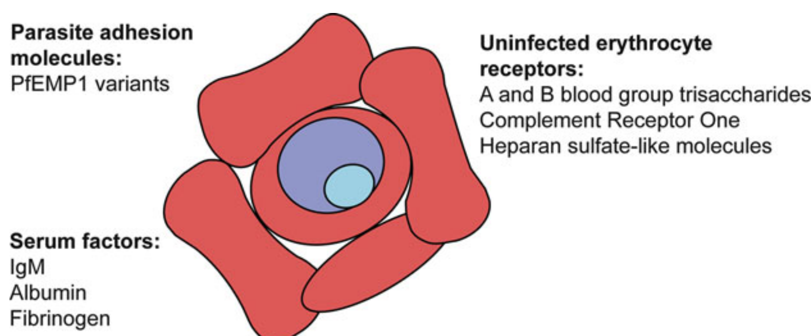

Fig. 5 Summary of *P. falciparum* rosetting mechanisms. Diagram to summarize the major parasite adhesion molecules, serum proteins, and uninfected erythrocyte receptors that have been shown to interact to bring about rosetting

erythrocytes (which lack the fucose residue normally present in group O) still form rosettes to the same rosette frequency as group A and B cells, indicating that other molecules are involved.

Complement receptor 1 (CR1) is known to be a receptor for rosetting, as parasites rosette poorly when grown in CR1-deficient erythrocytes (Rowe et al. 1997), and CR1 monoclonal antibodies (mAbs) and soluble recombinant CR1 inhibit rosetting in some, but not all, isolates (Rowe et al. 2000). In addition, a "heparan sulfate-like" molecule has been suggested as a rosetting receptor for some isolates; however, this remains poorly characterized (Vogt et al. 2004). Rarely, the

scavenger receptor CD36, which is expressed at low levels on mature erythrocytes, can act as a rosetting receptor (Handunnetti et al. 1992). CD36-dependent rosetting is a phenotype that has only been described to date in a single laboratory-adapted *P. falciparum* strain and has not been detected in other laboratory strains or clinical isolates. Recent work suggests that glycophorin C may also act as a receptor for rosetting (Niang et al. 2014; Lee et al. 2014).

Current data suggest that *P. falciparum* parasites form rosettes using more than one receptor on uninfected erythrocytes. For example, the laboratory-adapted *P. falciparum* clone IT4/R29 is blood group A preferring and CR1 dependent and binds “heparan sulfate-like” molecules (Fig. 4). Exactly which rosetting mechanisms are most important in clinical disease remains unclear, and it is possible that additional erythrocyte rosetting receptors remain to be discovered.

IgM and Other Serum Proteins

The complexity of the rosetting phenotype is emphasized further by the involvement of multiple serum proteins in *P. falciparum* rosetting. Some rosetting isolates bind “nonimmune” IgM from human serum/plasma onto the surface of infected cells, whereas others do not (Scholander et al. 1996). Initial data suggest that clinical isolates from children with severe malaria usually show the IgM-positive rosetting phenotype (Rowe et al. 2002b), although this needs further study. IgM-positive rosetting parasites express a distinct subset of PfEMP1 variants compared to IgM-negative rosetting parasites (Ghumra et al. 2012). The PfEMP1 variants from IgM-positive rosetting parasites bind directly to the Fc region of human IgM (Ghumra et al. 2008). IgM binding is thought to strengthen rosettes (Scholander et al. 1996), although the mechanism is unclear. IgM binding could also have other functions such as immune evasion by preventing specific acquired antibodies binding to PfEMP1, but this has not yet been tested in rosetting parasites.

Other serum/plasma proteins such as albumin and fibrinogen also enhance rosetting, although it is unclear whether this is due to specific interactions with molecules on infected erythrocytes, or more likely due to the nonspecific aggregating effect of these molecules on all erythrocytes (Treutiger et al. 1999). Roles for complement factor D and anti-B and 3 antibodies have also been proposed for one laboratory-adapted strain (Luginbuhl et al. 2007), although the relevance of these findings for *P. falciparum* isolates more widely is unknown.

Rosetting in Other *Plasmodium* Species

Rosetting has been described in all *Plasmodium* species that have been examined to date (Lowe et al. 1998). As with *P. falciparum*, the rosette frequency varies between isolates of a given species (Mackinnon et al. 2002). The PfEMP1 family is unique to *P. falciparum*; therefore, rosetting in other species must be mediated by different parasite molecules, with members of the *pir* variant surface antigen family being possible candidates (Cunningham et al. 2010). The other unique feature of *P. falciparum* rosetting is that infected erythrocytes can both rosette and cytoadhere to endothelial cells simultaneously, such that rosetting parasites are always sequestered and do not circulate in the peripheral blood (see Fig. 2). Rosetting is not specifically associated with life-threatening malaria in other *Plasmodium* species such as *P. chabaudi* and *P. vivax* (Mackinnon et al. 2002; Lee et al. 2014). It seems likely that it is the combination of rosetting, sequestration, and high parasite density that is responsible for the pathological effects of rosetting in falciparum malaria in humans. However, rosetting has been associated with anemia in vivax malaria (Marin-Menendez et al. 2013), and it is possible that *P. vivax* rosetting causes uninfected erythrocyte damage similar to that described for *P. falciparum* rosetting (Uyoga et al. 2012).

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Potential for Anti-Rosetting Interventions in Falciparum Malaria

Rosette-Disrupting Drugs

The strong evidence supporting a role for rosetting in the pathophysiology of severe falciparum malaria makes it a target for development of an adjunctive therapy for severe disease. Many patients with severe malaria currently die, despite treatment with rapidly acting and effective antimalarial drugs. There are no treatments available that target the underlying pathological processes in severe malaria, yet infected erythrocyte adhesion, microvascular obstruction, and downstream pathological processes can continue for many hours after the parasites have been killed (Hughes et al. 2010). An adjunctive therapy that could disrupt rosettes and relieve microvascular obstruction would have potential to improve the outcome of severe malaria.

Early work showed that heparin disrupts rosettes (Udomsangpetch et al. 1989), although it was not recommended for use in severe malaria patients due to concerns about its anticoagulant effects (Warrell 1997). A heparin derivative that has minimal anticoagulant action is currently undergoing clinical trials (Vogt et al. 2006). The disadvantage of heparin derivatives is that only some rosetting isolates (approximately one third to one half) are effectively disrupted by heparin (Carlson et al. 1992). Other sulfated glyconconjugate molecules, such as curdlan sulfate and fucoidan, have broader anti-rosetting effects (Kyriacou et al. 2007), but these molecules also have anticoagulant effects, raising safety concerns.

Other interventions targeting the uninfected erythrocyte receptors such as soluble CR1 or mAbs to CR1 are, like heparin, only effective against a subset of rosetting isolates (Rowe et al. 2000). While others, such as soluble blood group sugars, disrupt rosettes poorly or not at all (Carlson and Wahlgren 1992).

The complexity in the molecular mechanisms of rosetting described above may make it difficult to develop a single intervention that disrupts rosettes in all isolates. However, studies have begun to define the precise binding interaction sites between PfEMP1 and rosetting receptors on uninfected erythrocytes using X-ray crystallography (Juillerat et al. 2011; Vigan-Womas et al. 2012). Further work is needed to determine whether there are conserved binding sites in all *P. falciparum* rosetting isolates that could be targeted with small molecule drugs or biologics.

An Anti-rosetting Vaccine

Antibodies raised to rosette-mediating PfEMP1 recombinant proteins are highly effective at disrupting rosettes (Fig. 4) and can opsonize rosetting infected erythrocytes for phagocytosis (Ghumra et al. 2011; Ghumra et al. 2012). The presence of rosette-disrupting antibodies in humans is associated with protection from severe malaria (Carlson et al. 1990). Rosette-disrupting antibodies develop following childhood malaria infections in sub-Saharan Africa and may play a role in the rapid development of immunity to severe malaria in the first 5 years of life in endemic regions (Barragan et al. 1998).

Existing data suggest that antibodies to PfEMP1 are mostly variant- and strain-specific (Newbold et al. 1992). Surprisingly, antibodies against PfEMP1 variants that mediate IgM-positive rosetting have strain-transcending activity, recognizing *P. falciparum* laboratory-adapted strains and clinical isolates with the same rosetting phenotype (Ghumra et al. 2012). These data suggest the presence of conserved epitopes within rosette-mediating PfEMP1 variants from diverse parasite isolates. This raises the possibility of developing an anti-rosetting component of a malaria vaccine whose aim would be to prevent severe malaria.

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Future Research Directions

Current understanding of the molecular mechanisms of rosetting is based on detailed analysis of a small number of laboratory culture-adapted *P. falciparum* strains. Given the complexity in the phenotype that has been revealed by this work, further research is needed to understand which rosetting mechanisms are most important in severe malaria patients and to identify the best targets for interventions.

Cross-References

► 4-Hydroxynonenal in the Physiology and Pathology of Malaria
► Adjunct Therapies for Malaria
► Causes and Significance Malarial Retinopathy
► Cerebral Malaria: Pathophysiology of Clinical Features
► Cytoadherence
► Erythrocyte Deformability
► In Vitro Models of Disease
► Platelets and Their Role in Malaria Infections
► Red cells and Their Role in Malaria Infections
► The Contribution of *Post Mortem* Studies to Understanding the Pathophysiology of Malaria
► The Pathophysiology of Hypoglycaemia and Lactic Acidosis in Malaria

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