Cell–cell interaction in the pathogenesis of severe falciparum malaria

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ABSTRACT – One of the major unresolved questions in malaria is why some patients with *Plasmodium falciparum* infection become so sick and die. Cell–cell interactions between the parasite and the host involving adherence/invasion appear generally, but not exclusively, to correlate with severity. The most important of these interactions in the asexual blood cycle are: (i) the invasion of red cells by merozoites, (ii) the binding of parasitised red blood cells (PRBC) to uninfected red cells (rosetting), (iii) the binding of PRBC to endothelial cells in critical organs (cytoadherence) and (iv) the induction of pro-inflammatory cytokines by PRBC, notably tumour necrosis factor (TNFα). The resulting clinical manifestations are protean. Analysis of these cellular interactions has revealed marked heterogeneity in molecular specificity which highlights the complexity of pathogenesis, but also opens the way to new modalities for treating this deadly infection.

Over a third of the world's population is at risk for malaria and up to 250 million clinical cases are said to occur annually. Of the four species of malaria parasites known to infect man, *Plasmodium falciparum* is responsible for over one million deaths each year[1]. Why do some patients develop severe disease, become very sick and die? *P. falciparum* is a eukaryote with a genome of about 30 million base-pairs, considerably larger, for example, than that of the bacterium *Haemophilus influenzae B* (1.8 million base-pairs) or the hepatitis B virus (3,500 base-pairs), for which successful vaccines exist. Almost no other pathogen in man invades the red cell, which is the part of the life-cycle responsible for disease and may account for the unusual pathogenesis of malaria. The parasitised red cell (PRBC), the unit of pathology, is unlike a red cell or parasite and possesses distinctive biological properties. At any one time many people living in an endemic area are infected with malarial parasites but remain asymptomatic. Only a minority develop symptomatic disease, and even fewer progress to severe disease with the well-recognised but extensive range of features which include cerebral malaria, hypoglycaemia, respiratory distress, circulatory collapse, fluid and electrolyte imbalance, blackwater fever, renal failure, disseminated intravascular coagulation and anaemia, amongst others[2]. Whilst these clinical features are familiar to many, our understanding of the underlying cellular interactions between host and parasite remains imperfect.

Parasite–host interactions

The pathogenesis of malaria is clearly the result of complex interactions between the parasite, host and environment. Crucial specific interactions occur between parasite and host (Fig 1), including the invasion of uninfected red cells (invasion), the binding of PRBC to uninfected red cells (rosetting) or to endothelial cells in the deep tissues (cytoadherence), and the production of a 'toxin' or 'toxins' by the parasite leading to the release of numerous pro-inflammatory cytokines such as tumour necrosis factor (TNFα). More detailed reviews on pathophysiology can be found elsewhere[3,4].

Invasion

The invasion of the red cell (and occasionally platelets), by the parasite is paramount to our understanding of the pathogenesis of malaria[5]. Invasion is a highly specific, ordered and sequential process lasting about 30 seconds. The merozoite attaches to a susceptible red cell, re-orientates itself so that its apical end is apposed to the red cell membrane, and then slowly moves into a localised invagination of the red cell which subsequently envelops it as the parasitophorous vacuolar membrane (PVM).

The red cell membrane poses a formidable barrier to invasion; few organisms other than malarial parasites are capable of penetrating it. Identification of the molecules on the red cell surface to which merozoites bind has been the subject of intensive research (Table 1). The red cell sialoglycoproteins or 'glycophorins' (GPs) especially GPA and GPB, were the first receptors identified[6]. Of the sites involved on GP, the O-linked tetrasaccharides, rather than the much larger N-linked sugars on these molecules, were found to be important. The resistance of the Tn red cell phenotype to invasion (Tn cells lack a glycosyl transferase which results in cells deficient in

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sialic acid), and the sensitivity of invasion to neuraminidase/sialidase, led to the identification of sialic acid on GP as a major recognition site on these O-linked sugars[7]. Invasion of uninfected cells correlates with the binding of a conserved parasite molecule, the erythrocyte binding antigen of 175 kDa (EBA 175). EBA 175 recognises sialic acid linked in an α2-3 configuration to galactose, but not in an α2-6 configuration to N-acetyl galactosamine[8]. It had been suggested that the malaria parasite produced a sialidase, but a potent inhibitor of influenza virus sialidase, 4-guanidino-2,3-didehydro-D-N-acetyl neuraminic acid (4-guanidino Neu5Ac2en), better known as zanamavir (Relenza™), failed to retard parasite multiplication, nor were parasites found to possess sialidase or trans-sialidase activity[9]. However, there are also *P. falciparum* parasites capable of invading cells devoid of GPA or GPB by sialic acid-independent pathways. In a recent study from India, the latter appeared to be more commonly used by parasites obtained directly from patients[10].

Once attachment and orientation have occurred, interiorisation accompanied by deformation of the red cell membrane follows, but the multiplicity of molecules involved in this part of the process and their individual roles have not as yet been ascertained[11]. When red cell deformability was measured by ektacytometry (which examines how cells deform when exposed to shear stress) and lateral mobility of red cell transmembrane proteins such as GP and Band 3 by the method of fluorescence recovery after photobleaching (FRAP), it was found that alterations in the physicochemical properties of the red cell membrane also play an important role in regulating invasion[12].

Relationship of parasite invasion to pathogenesis

Within a given individual, the severity of malarial disease relates to parasite density. This in turn relates to the ability of the parasite to invade and multiply within red cells. It is only recently (largely because of technical difficulties) that parasite multiplicative ability within red cells has been shown to relate to disease severity[13]. *P. falciparum* isolates from patients with severe malaria were found to possess initial *in vitro* multiplication rates three times higher than those from patients with uncomplicated malaria. They also exhibited apparently unrestricted red cell invasion (ie could invade all the cells present), whereas isolates from uncomplicated cases were calculated (by observing the frequency of multiply infected cells) to be restricted to 40% of red cells. Unfortunately, these invasion rates were not tested under conditions of flow when invasion rates are generally lower and might have been even more pronounced in differentiating 'virulent' from 'less virulent' parasites. The ability of *P. falciparum* to invade cells of all ages, in contrast to the reticulocyte restriction of *Plasmodium vivax*, enhances its multiplicative ability, even though *P. falciparum in vitro* and in mild infections maintains a predilection for younger red cells[14]. The ability to invade red blood cells may yet prove to be a crucial factor in the pathogenesis of falciparum malaria.

Rosetting

In rosetting, an interaction distinct from cytoadherence, mature PRBCs bind uninfected red cells to their surface. Interest in rosetting derived from its association with severe disease. All

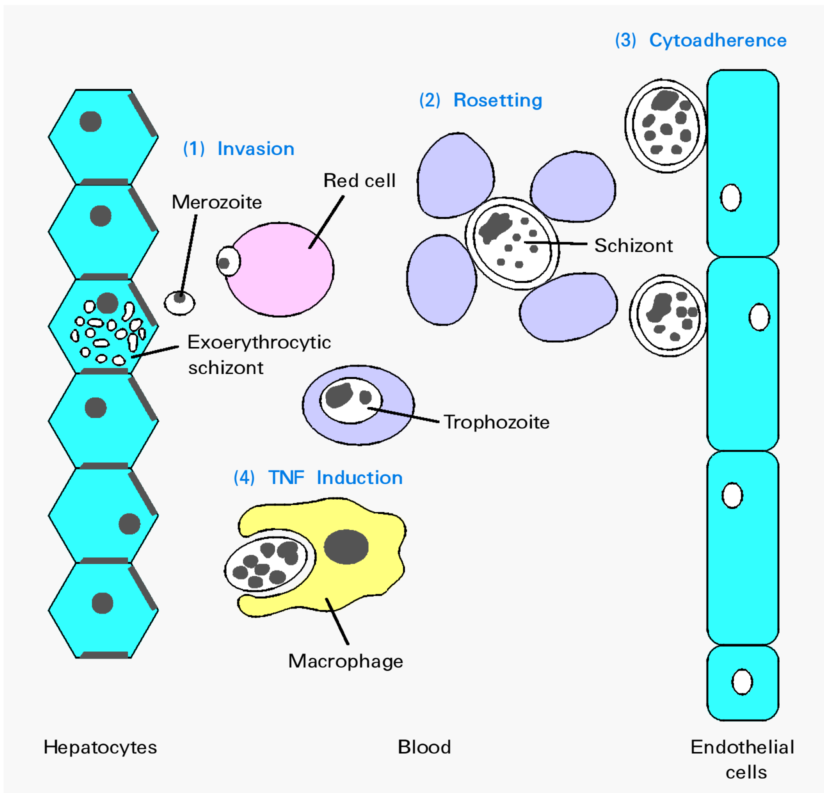

Fig 1. Major cell–cell interactions in the pathogenesis of falciparum malaria.

isolates of *P. falciparum* obtained from children with cerebral malaria in the Gambia were capable of rosetting, whereas many isolates from patients with mild disease were not[15]. Moreover, plasma from children with mild disease could more often disrupt preformed rosettes than plasma from patients with cerebral malaria. The anti-rosetting activity was present in the immunoglobulin (Ig) fraction.

The definitive molecular components of rosetting on the infected and uninfected red cell have yet to be resolved. A number of high molecular weight proteins protrude from the so-called 'knobs' on the PRBC surface, of which the best known is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1). This molecule shows variation in size and antigenic properties and may be involved in a variety of adhesive interactions. Whilst one group has implicated PfEMP-1 and CD36 on infected and uninfected red cells, respectively[16], another has proposed small molecular weight molecules, the 'rosettins' on the infected cell and the ABO blood group sugars (especially A) on the uninfected cell[17]. The complement receptor 1 (CR1) is a further candidate on red cells for rosetting[18].

Most of the studies of the correlates of rosetting and disease have focused on the PRBC and the uninfected red cell. However, several parasite lines or isolates have now been found to be specifically dependent on human serum/plasma[19]. Multiple serum components are involved in rosetting, including IgM (non-immune) and at least two others[20], one of which may be fibrinogen. Using a conconavalin A column which binds mannose-containing molecules, neither the bound nor unbound fractions could restore rosetting when used alone, but the two fractions mixed together could do so. IgM stabilised rosette formation but could not return rosetting to control levels.

The relationship of rosetting to pathogenesis

The mechanisms by which rosetting leads to disease remain obscure. Rosette formation has not convincingly been observed *in vivo*. Perfusion of the rat mesoappendix with rosetting parasites resulted in greater vascular resistance than with non-rosetting parasites[21]. An alternative mechanism is that rosette

Table 1. Some of the parasite and host molecules involved in the specific cellular interactions in falciparum malaria.

	Invasion	Rosetting	Cytoadherence	TNF induction
Parasite-induced molecules	Erythrocyte binding antigen (EBA-175) Merozoite surface protein-1 (MSP-1) MSP-2/4/5 Apical membrane antigen (AMA) Rhoptry associated protein (RAP-1) RAP-2 RAP-3	Plasmodium falciparum erythrocyte membrane-1 (PfEMP-1) Rosettins	PfEMP-1 PfEMP-3 Histidine-rich protein-1 (HRP-1) Ring surface protein 1+2 Modified band-3 (Pfalhesin) Cytoadherence-linked asexual gene protein (CLAG)	Glycosylphosphatidylinositol-anchored molecules Phospholipid Haemazoin
Molecules of host cell origin	Glycophorin A (Gp-A) Gp-B Gp-C (Sialic acid a2-3 linkage) Blood group A Sialic acid independent pathways – as yet unspecified	CD36 Rosettins Intercellular adhesion molecule (ICAM-1) Complement receptor-1 (CR1)	CD36 Thrombospondin (ICAM-2) Vascular cellular adhesion molecule (VCAM-1) Platelet endothelial cellular adhesion molecule (PECAM-1) E-selectin P-selectin Chondroitin sulphate A (CSA) Hyaluronic acid (HA) Heparan sulphate-like glycosaminoglycan (GAG) $\alpha_{\upsilon}\beta_3$ integrin	CD36 on monocyte/ macrophages Red cell membrane
Molecules in the host serum/plasma	Immunoglobulin Immune immunoglobulin Other undefined factors	Non-immune IgM	Immune immunoglobulin	Unknown

Key Points

The pathogenesis of falciparum malaria is complex; the unit of pathology is the parasitised red blood cell (PRBC)

Since the PRBC circulates to almost every organ in the body, the clinical manifestations of severe disease are wide-ranging

Invasion of red cells, rosetting, cytoadherence and the induction of pro-inflammatory cytokines are central to pathogenesis

For each of these cell–cell interactions there are specific ligands and receptors in both parasite and host; the adherent/invasive phenotype generally correlates with disease severity

Understanding pathogenesis should lead to advances in the management of severe malaria

formation, by juxtaposing infected and uninfected cells, might enhance invasion and parasite multiplication especially under conditions of flow. Rigorous experiments with a rosetting and non-rosetting parasite line, under static conditions or with red cells maintained under conditions of flow (ie in suspension), demonstrated that rosetting neither increases invasion nor targets merozoites within a rosette into adjacent uninfected cells[22]. Rosetting may perhaps reflect the phenotypic expression of some other parasite property such as adherence to a specific cell type, which in turn relates to pathogenesis.

Cytoadherence

Cytoadherence is the process whereby mature PRBCs specifically bind to endothelial cells in post-capillary venules, which explains why PRBCs are seldom observed in peripheral blood samples. On scanning electron microscopy, a number of regular, symmetrically arranged 'knobs' appear on the surface of the infected cell as the parasite of *P. falciparum* matures. These knobs are thought to be the site at which the parasitised red cell attaches to endothelial cells in the deep tissues, although parasites without knobs are capable of cytoadherence *in vitro*. With maturation of the parasite, the red cell becomes spherical and less deformable. Cytoadherence has been the cell interaction in malaria most studied, so it is not surprising that a multiplicity of molecules on both parasitised red cell and endothelial cell have been identified that mediate binding (Table 1)[23]. PfEMP-1 (molecular weight 200-350 kDa) appears to be the most important on the mature PRBC, but recently another group of smaller molecules, the ring surface proteins (RSP 1 and 2), has been identified on immature PRBC[24]. It is thus conceivable that not only is the degree to which PRBCs cytoadhere important in pathogenesis, but also the stage in the erythrocyte life cycle when cytoadherence commences.

Numerous molecules are involved in cytoadherence (allowing for a wide heterogeneity in binding), the relative roles of which have yet to be established. PfEMP-1 is of particular interest because the amino-terminal head structure of this molecule – which includes a Duffy-like binding domain and a cysteine-rich interdomain region – appears to mediate adherence to a diverse set of host receptors, including platelet-endothelial cell adhesion molecule (PECAM), the blood group A antigen, non-immune IgM, a heparan sulphate-like glycosaminoglycan and CD36[25] (Table 1). ICAM-1, VCAM-1 and E-selectin, but not CD36 and thrombospondin, are upregulated by cytokines[26]. Higher levels of ICAM-1 and E-selectin were detected by immunochemistry on vessels in the brain of patients with severe malaria than in uninfected controls, whereas endothelial CD36 and thrombospondin showed no increase[27]. At the sites of sequestration there was co-localisation of ICAM-1, CD36 and thrombospondin but no evidence of a cellular inflammatory response. In malaria, ICAM-1 acts as a rolling receptor, whereas CD36 and thrombospondin seem to be involved in more stable interactions[28], the reverse of that used by neutrophils.

The relationship of cytoadherence to pathogenesis

Whatever the specific mechanisms for cytoadherence, a more pertinent question is the significance of cytoadherence in pathogenesis. By immobilising parasites in various organs, cytoadherence will prevent their passage through the spleen, a major site of parasite destruction, localise maturing parasites at sites of reduced oxygen tension which favours parasite growth, and may facilitate invasion of uninfected red cells. In the process of cytoadherence, parasites may produce damage in a number of ways, although there may not always be an association between cytoadherence and severe disease[29]. Cytoadherent parasites presumably lead to microvascular obstruction, although the relevance and extent of this obstruction in pathogenesis remains unclear. Total cerebral blood flow in cerebral malaria remains unaltered whilst tissue infarction and necrosis, an expected sequel of microcirculatory blockage, is uncommon. Cytoadherence, a feature of falciparum malaria, may lead to local endothelial cell activation, release of cytokines and subsequent damage to adjacent tissues, unlike in non-falciparum malaria in which cytoadherence is thought not to occur.

Induction of pro-inflammatory cytokines

The acute rigor, so characteristic of malaria and occurring at the time of schizont rupture, is highly suggestive of a physiologic response to a toxin. However, attempts to identify a definitive malarial 'toxin' remain unresolved. The glycosylphosphotidylinositol (GPI)-anchored molecules of the parasite are favoured candidates[30], but other molecules have been proposed including as yet undefined phospholipid molecules[31] and a protease-sensitive component of malarial pigment[31]. Even products from lysed uninfected red cells are capable of inducing cytokine release, although at least 200 times less so than parasitised cells[32]. Much of the research into such a toxin before 1998 was put into question when it was found that most long-term laboratory cultures of parasites were contaminated with

mycoplasma, a potent inducer of TNF[33]. Mycoplasma-free parasite lysates were almost devoid of TNF-inducing activity. Optimal induction of TNFα by *P. falciparum* requires the rupture of live parasites in close proximity to responding peripheral blood mononuclear cells (O'Dea, in preparation). Such findings have important implications in the understanding of malarial pathogenesis as they emphasise the importance of site-specific sequestration, question the relevance of measuring plasma TNF levels and complicate any therapeutic intervention based on the reduction of excessive pro-inflammatory cytokine stimulation.

Tumour necrosis factor induction and pathogenesis

Whatever the mechanism of TNF induction, there is certainly a good correlation between high levels of TNF and the outcome of severe falciparum malaria[34]. TNF levels are highest (>250 pg/ml) in those children who died or developed neurological sequelae. Excessive TNF production and release could account for the fever, leukocytosis, enhanced sequestration, hypoglycaemia, dyserythropoiesis, and possibly even the impaired consciousness observed in severe disease. Other cytokines such as many of the interleukins and γ-interferon also play a synergistic/antagonistic role. It is therefore disappointing that treatment with anti-TNF antibodies does not have any impact on severe disease other than a reduction in fever[35].

Host genetic heterogeneity in the TNF response by a given individual may have a major effect on outcome. Homozygotes for the TNF2 allele (a polymorphism located at position –308 upstream from the start of the TNF gene) were found to have an almost eight-fold risk of death and severe neurological sequelae from cerebral malaria. Such a polymorphism has been associated with higher constitutive and inducible levels of TNF. It has been invoked in a compelling hypothesis of why inflammatory conditions such as systemic lupus erythematosus (SLE) and sarcoidosis are uncommon in individuals living in malarial endemic areas, but become increasingly frequent in those who migrate to temperate climates where there is no malaria[36]. Malaria is immunosuppressive and reduces the likelihood of lupus nephritis in animal models. As it is often a chronic infection, malaria may maintain macrophage activation at levels sufficient to suppress an autoimmune response in susceptible individuals. Natural selection may have favoured individuals with a reduced pro-inflammatory cytokine response that could reduce the risk of death from malaria. When such individuals migrate to non-malarious areas, lower constitutive or induced TNF secretion might place them at higher risk of conditions such as SLE or sarcoidosis. Cogent as such an argument might seem, it remains at present only hypothetical.

Conclusions

Parasite–host interactions are important in the pathogenesis of severe falciparum malaria. Four (ie invasion, rosetting, cytoadherence and TNFα induction) have been highlighted, and there may be more in this complex parasite–host interaction. There is a high degree of parasite–host molecular specificity in these interactions and no doubt more will emerge. The parasite can use a number of these pathways; if one is unavailable for whatever reason, the parasite will adapt and use alternative means to reach the same end. The parasite clearly makes a substantial investment to achieve this. The relative importance *in vivo* of these different pathways in each of the four interactions described has yet to be established. The general ability to bind or invade seems to be associated with virulence. These observations will have major implications in designing vaccines and therapeutic interventions for this devastating disease.

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