Review TRENDS in Parasitology Vol.20 No.12 December 2004

Clinical features and pathogenesis of severe malaria

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A major change in recent years has been the recognition that severe malaria, predominantly caused by *Plasmodium falciparum*, is a complex multi-system disorder presenting with a range of clinical features. It is becoming apparent that syndromes such as cerebral malaria, which were previously considered relatively clear cut, are not homogenous conditions with a single pathological correlate or pathogenic process. This creates challenges both for elucidating key mechanisms of disease and for identifying suitable targets for adjunctive therapy. The development of severe malaria probably results from a combination of parasite-specific factors, such as adhesion and sequestration in the vasculature and the release of bioactive molecules, together with host inflammatory responses. These include cytokine and chemokine production and cellular infiltrates. This review summarizes progress in several areas presented at a recent meeting.

The vast majority of malaria cases present as nonspecific febrile illnesses that are relatively easily terminated by either antimalarial treatment or, eventually, by host responses. Although only a minority of cases – ~1% – progress to severe life-threatening disease, the absolute number is massive, with around one million deaths each year in sub-Saharan Africa alone [1,2]. The key to reducing this toll must lie with better prevention; however, even if the most optimistic predictions for reaching current global targets are achieved, severe malaria will remain as one of the most common life-threatening illnesses in the tropics. Improvements in outcome will only come from a better understanding of the underlying processes that result in severe manifestations and death. This review summarizes progress in several areas presented at the recent Molecular Approaches to Malaria meeting*.

Clinical features and pathogenesis of severe malaria

For many years, severe malaria was pictured as essentially two major syndromes, with relatively simple underlying pathogenic processes: (i) severe anaemia caused by the destruction of red blood cells (RBCs); and (ii) cerebral malaria (CM) caused by obstruction of small vessels of the brain by sequestered parasites. A major change in recent years has been the recognition (some would say the re-recognition) that severe malaria is a complex multi-system disorder with many similarities to sepsis syndromes. At the clinical level, this is evident in the recognition of metabolic acidosis (leading to the clinical picture of respiratory distress) as the strongest predictor of death in severe malaria [3–5]. The pathogenesis of metabolic acidosis is poorly understood. Hypovolaemia [3,6] is a major feature of severe malaria and, when further exacerbated by anaemia and microvascular obstruction from sequestered parasites, is likely to lead to decreased delivery of oxygen to tissues, anaerobic metabolism and lactic acidosis. However, this is undoubtedly an oversimplification: in many cases, there is no hyperlactataemia. Clark and Cowden [7] have pointed out that, as in sepsis, cytokine-induced failure of oxygen utilization is likely to play an important role. Indeed, immunopathogenic processes are now recognized as having a central role in severe malaria, with proinflammatory cytokine cascades leading to complex downstream metabolic changes. It has become apparent that even syndromes previously considered 'clear cut', such as CM, are not homogenous conditions with a single histological correlate [8]. (Current understanding of the pathogenesis of severe malaria is comprehensively reviewed in Ref. [7].)

Traditionally, approaches to understanding pathogenesis have involved clinical studies in humans, experimental infections in animal models and *in vitro* modelling of pathogenic processes (Table 1). More recently, functional genomics has provided a new approach to examining multiple pathogenic processes in an integrated way. Christian Ockenhouse *et al.* (Walter Reed Army Institute of Research, Silver Spring, MD, USA) presented data from screening arrays of more than 23 000 human genes at different stages of human malaria infections. Expression of co-regulated genes fell into functional groups, such as erythrocyte-associated genes and immune-response genes, regulation of which is related to homologous sequences upstream in promoter regions of target genes. It was interesting how early in the disease process the changes in gene expression were seen; indeed, most upregulation would precede the onset of clinical symptoms. Jürgen Kun *et al.* (University of Tübingen, Tübingen, Germany) reported that differential expression of two relatively small sets of genes distinguished children with severe malaria from those with mild disease. The challenge now is to simplify the complexity that results from such

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Available online 7 October 2004
* The Molecular Approaches to Malaria (MAM) meeting was held 1–5 February 2004 in Lorne, Australia.

 1471-4922/$ - see front matter doi:10.1016/j.pt.2004.09.006

Table 1. Clinical features of malaria and possible mechanisms of disease

Syndromes	Clinical features	Disease mechanisms[a]	Refs
Severe anaemia	Shock; impaired consciousness; respiratory distress	Reduced RBC production (reduced erythropoietin activity, proinflammatory cytokines); increased RBC destruction (parasite-mediated, eythrophagocytosis, antibody and complement-mediated lysis)	[86–92]
Cerebral complications (cerebral malaria)	Impaired consciousness; convulsions; long-term neurological deficits	Microvascular obstruction (parasites, platelets, rosettes, microparticles); proinflammatory cytokines; parasite toxins (e.g. GPI)	[8,10–13,26,29,33–36, 39–41,47,48,62–72]
Metabolic acidosis	Respiratory distress, hypoxia, tachypnea; acidaemia; reduced central venous pressure	Reduced tissue perfusion (hypovolaemia, reduced cardiac output, anaemia); parasite products; parasite products; proinflammatory cytokines; pulmonary pathology (airway obstruction, reduced diffusion)	[3–7,17–19]
Other	Hypoglycaemia; disseminated intravascular coagulation	Parasite products and/or toxins; proinflammatory cytokines; cytoadherence	[7,9,17–20,23–25,27,62]
Malaria in pregnancy	Placental infection; low birth weight and fetal loss; maternal anaemia	Premature delivery and fetal growth restriction; placental mononuclear cell infiltrates and inflammation; proinflammatory cytokines	[14,15,42–45,49,80–85]

[a]Abbreviations: GPI, glycosylphosphatidylinositol; RBC, red blood cell.

approaches and to generate testable hypotheses that can potentially lead to improved prevention or treatment.

Inflammatory processes and immunopathology

Cytokines

Proinflammatory cytokines have long been implicated in the pathogenesis of CM, and anti-inflammatory cytokines, such as interleukin-10 (IL-10), have been proposed to have a protective or counter-regulatory role [7]. Tumour necrosis factor (TNF) is raised in those with severe malaria [9–11] and has been implicated in the pathogenesis of murine CM [12]. Mice deficient in tumour necrosis factor receptor 2 (TNFR2), a receptor for TNF, are resistant to CM [13]. TNF is also raised in placental malaria and is associated with low birth weight [14,15]. However, Clark and Cowden [7] have pointed out that the apparent centrality of TNF might be misleading, and could be a reflection of its ease of measurement, with other cytokines being potentially as important. Recent studies by Christian Engwerda and colleagues (Queensland Institute of Medical Research, Australia), strongly suggest that murine CM requires lymphotoxin α (LTα) rather than TNF [16]. Mice deficient in LTα were resistant to CM, whereas those deficient in TNF remained susceptible. LTα probably also binds to TNFR2. Many immunological reagents used to measure TNF also react against LTα. The involvement of TNF in severe human malaria suggested in earlier studies [17,18] might need to be re-assessed. The proinflammatory cytokine interferon γ (IFN-γ) has been associated with both pathogenesis and protection against human disease [19,20]. In a murine model of severe malaria, IFN-γ production by natural killer (NK) T cells has been implicated in disease pathogenesis (Diana Hansen; Walter and Eliza Hall Institute, Victoria, Australia), whereas production of IL-4 by NKT cells was associated with protection against severe disease [21]. IFN-γ receptor (IFN-γR)-knockout mice were resistant to CM, but remained susceptible to severe malaria and death [22]. It is likely that the balance and timing of proinflammatory and anti-inflammatory cytokine production are important in disease and parasite clearance. Andrew Mitchell (University of Sydney, Sydney, Australia) reported that an early (24 h) surge of IFN-γ in mice infected with *Plasmodium berghei* 173 was associated with protection from CM, whereas a late surge (3 days) in *P. berghei* ANKA was associated with immunopathology. Much interest is now focused on the downstream events triggered by proinflammatory cytokines, and agents that favourably modify cytokine production might be useful adjunctive therapies in reducing the mortality and long-term sequelae of severe malaria.

Nitric oxide

Population studies generally support an association between protection from severe malaria and nitric oxide (NO) production, measured indirectly by examining NO metabolites, NO synthase 2 (NOS2) expression in peripheral blood monocytes and NOS2 promoter polymorphisms [23–25]. NO synthesis requires extracellular arginine, and recent studies found an association between hypoargininaemia and severe malaria and death in children [26]. This might be consistent with a protective role of NO against severe malaria, or hypoargenininaemia might have resulted from activation of iNOS in severe malaria. However, immunohistochemistry of cerebral tissue post-mortem revealed increased inducible NOS expression and markers of NO production in severe malaria [27]. In addition, NO has been implicated in the pathogenesis of severe sepsis, and it has been suggested that NO could alternatively play a role in the pathogenesis of severe disease [28]. Nick Anstey *et al.* (Menzies School of Health Research, Darwin, Australia) propose that, when low arginine limits NO production, it also favours the generation of oxidants that might contribute to pathogenesis, potentially explaining differences in published findings. The administration of systemic arginine might be a beneficial adjunctive therapy in the management of severe malaria, or have a role in preventing severe malaria; this is an approach that should be tested in clinical trials.

The kynurenine pathway

Levels of the excitotoxic metabolite quinolinic acid were first reported to be elevated in the cerebrospinal fluid of Kenyan children with severe malaria by Dobbie [29] and a similar finding has since been reported in Malawian children [30,31]. However, in Vietnamese adults, the ratio

of concentration of quinolinic acid to the neuroprotective antagonist kynurenic acid was not raised, and no correlation with depth of coma or convulsions was found [31]. In recent studies in the *P. berghei* ANKA model, levels of indoleamine 2,3-deaminase (IDO), the rate-limiting enzyme in the kynurenine pathway, were increased 40 times compared with controls, at the point of developing cerebral symptoms. IFN-γ is the main inducer of IDO, and IFN-γ-knockout mice showed no rise in IDO and were protected against CM (Nick Hunt; University of Sydney, Sydney, Australia).

Other mediators

Recent studies have provided strong evidence supporting a role for perforin in the pathogenesis of severe murine malaria, through disruption of the blood–brain barrier (Sarah Potter; University of Sydney, Sydney, Australia) [32]. Although perforin has not yet been studied in human disease, mice deficient in perforin appear to be resistant to cerebral and severe complications of malaria. $CD8^+$ T cells have been implicated in the pathogenesis of murine CM [33] and might be a source of perforin, as might NKT cells. Changes in prostaglandin synthesis [34,35] and expression of chemokines (Jenny Miu; University of Sydney, Sydney, Australia) have also been implicated in disease pathogenesis in mice and to a lesser extent in a protective role in humans [36]. It remains to be established how these changes relate to one another in the causal pathway, and to what extent these processes contribute to human severe malaria. Finally, intriguing evidence for a further downstream pathogenic effect of cytokines was summarized by Georges Grau and Valéry Combes (Université de la Méditerranée, Aix-Marseille, France). Cytokine stimulation often leads to membrane vesiculation, and levels of endothelial microparticles were markedly raised in Malawian children with severe malaria and correlated with plasma TNF concentrations. In parallel studies in mice, animals genetically deficient for the *ABCA1* gene, and therefore unable to produce microparticles, were resistant to CM. It is hypothesized that endothelial microparticles could contribute directly to pathogenesis, possibly by increasing vascular plugging and interaction with immune cells.

The triggers that lead to excess proinflammatory cytokines are not well understood, but glycosylphosphatidylinositol (GPI) of *Plasmodium falciparum* has been implicated in several studies. GPI can stimulate TNF production by macrophages and increase iNOS expression [37]. Furthermore, vaccination with the synthetic glycan moiety of GPI protected mice from severe complications of experimental malaria [38].

Sequestration

Post-mortem examinations of people who have died from *P. falciparum* infection show accumulation of parasitized RBCs (pRBCs) within the small vessels of many tissues [39,40]. This phenomenon is likely to be important by contributing to high total body parasitaemias and focusing pathology in specific sites. Several post-mortem studies have demonstrated greater pRBC sequestration in the brain compared with other organs [40,41] in individuals dying of CM. However, as reviewed by Ref. [7], establishing a direct cause-and-effect relationship between sequestration and CM has proven difficult. This is likely to be owing, in part, to the heterogeneity of pathogenic processes that can lead to the single end point of coma, as indicated in clinical studies [4] and recently supported by detailed histological studies in Malawian children [8]. During pregnancy, pRBCs typically sequester in the placenta [42,43] and maternal malaria is associated with intra-uterine growth retardation and premature delivery, resulting in neonatal mortality [44]. Maternal health also suffers through the development of maternal anaemia and the resultant increased likelihood of maternal death, recently reviewed by Shulman and Dorman [45].

The consequences of sequestration to the host are thus variable and can be severe. Although the link between sequestration and clinical disease in humans remains indirect [46], a degree of obstruction to blood flow will occur in vasculature (where this phenomenon occurs). This could lead to hypoxia, reduction of metabolite exchange and the release of inflammatory mediators. In the brain, it could contribute directly to cerebral oedema and raised intracranial pressure [47].

Cytoadherence

Sequestration occurs principally during the second half of the intra-erythrocytic asexual growth phase of the parasite, following the adherence of mature parasites [48,49] to endothelial cells through electron-dense knobs on the pRBC surface [40]. *In vitro* studies have identified several cell-surface molecules as potential receptors for pRBC binding, including thrombospondin (TSP), CD36, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule, E-selectin, chondroitin sulphate A (CSA), CD31 and hyaluronic acid (HA) [50–57]. In addition to adhering to endothelial cells and syncytiotrophoblasts, mature-stage pRBCs can also adhere to non-infected RBCs [58], forming rosettes, and to other pRBCs, forming clumps (with platelets) or autoagglutinates [59,60].

Linking specific cytoadherence phenotypes to clinical syndromes has proved difficult. A plausible case can be made for ICAM-1 as a key host receptor in the brain: it is widely distributed on cerebral vessels, is upregulated by cytokines including TNF-α and was co-localized with pRBCs in brains of patients dying of CM [61]. However, parasites from the peripheral blood of children with CM have not been consistently found to have higher ICAM-1 binding [62,63]. Although it is likely that the association is diluted by the heterogeneity of clinical cases of CM, there might also be heterogeneity in parasites themselves. Man Tsuey Tse and colleagues (University of Liverpool, Liverpool, UK) have used a range of mutant ICAMs to show that different parasite variants use different residues on the ICAM-1 molecule.

Another major endothelial receptor, CD36, is not detected on human cerebral vasculature [61], but is ubiquitously expressed in lung, kidney, liver and muscle vasculature. Most parasite isolates causing clinical disease in non-pregnant individuals can bind to CD36 [63,64]. The relationship between CD36 binding and pathogenesis is not clear, with alternative lines of evidence supporting

contradictory models [63–66]. Clumping of pRBCs, which is associated with severe disease, is predominantly mediated by CD36 expressed on platelets [59], but parasites from children with CM tend to have relatively lower CD36 binding [63,64]. Intriguing possible explanations for some of these discrepancies were presented by two groups. Georges Grau *et al.* had previously reported that platelet accumulation in cerebral microvessels was significantly greater in children who had died from CM compared with those who had died as a result of severe malarial anaemia or a non-malaria encephalopathy [67]. They now report that platelets are able to bind to CD36-deficient endothelial cells that have been stimulated with TNF and then act as a bridge to infected erythrocytes. This might allow cytoadherence of parasites to platelet-expressed-CD36 in areas of the vasculature, such as the placenta and cerebral microvessels, where CD36 expression is low [68]. In malarious areas of the Western Pacific, Southeast Asian ovalocytosis (SAO) is a genetic trait that occurs at high frequencies and provides significant protection against CM [69]. Alfred Cortés (Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea) reported that, under flow conditions, parasitized SAO RBCs bound at significantly higher levels to CD36 compared with non-SAO pRBCs. They suggest that the protection afforded to SAO individuals might be caused by an altered distribution of sequestered parasite mass, with pRBCs preferentially binding to CD36 as opposed to ICAM-1, and thus avoiding the cerebral vasculature

Rosetting presents a second example of where effects of host genetic polymorphisms could explain previously discordant results on the relationship between cytoadherence phenotype and disease. Most studies in Africa have shown an association between the rosetting phenotype and disease severity [70], which has been absent in studies from Melanesia [71]. Ian Cockburn (University of Edinburgh, Edinburgh, UK) added to this intriguing debate by discussing the following findings: one of the key RBC receptors for rosetting is CR1; CR1 deficiency is common in Melanesian populations and is associated with protection from severe malaria [72]. This strengthens the evidence for an association between rosetting and disease severity.

The relationship between cytoadherence phenotype and disease expression might be further complicated by interactions between pRBCs and host cells. S. Chakravorty (Liverpool School of Tropical Medicine, Liverpool, UK) reported that pRBCs (and to a lesser extent, non-infected RBCs) increased ICAM-1 expression by endothelial cells *in vitro*, and that this expression was increased further in the presence of low concentrations of TNF, with the combination of pRBCs and TNF inducing significantly more ICAM-1 expression than either alone.

Parasite adhesion molecules and disease phenotype

To date, most cytoadherence phenomena appear to be mediated by PfEMP-1, a high-molecular-weight protein of approximately 240 kDa encoded by the *var* gene family [73–75], and inserted into the erythrocyte membrane between 16 and 20 h after invasion. There are about 60 copies of *var* genes within each haploid *P. falciparum* genome, and these are situated predominantly in the subtelomeric regions of all chromosomes [76,77]. The highly variable extracellular region consists of several domains that have homology with the Duffy binding ligand of *Plasmodium vivax* [named Duffy-binding-like (DBL) domains] [78], and cysteine-rich interdomain regions (CIDR). Although similar in terms of overall structure, each PfEMP-1 is highly polymorphic in sequence. PfEMP-1 has been shown to bind to many host receptors (Figure 1). It is hoped that there might be a restricted number of PfEMP-1 molecules expressed in severe disease, or relatively conserved receptor-binding sites, and that these could be suitable vaccine targets. Recent studies by Anja Jensen *et al.* (University of Copenhagen, Copenhagen, Denmark) suggest that a particular group of *var* genes (classified as type A) might be commonly expressed or expressed in association with severe disease [79]. Despite the potential diversity of PfEMP-1, it was also found that, within one *var* gene, a substantial proportion of exposed children and adults specifically had antibodies to one recombinant DBL domain over the others (Claire Mackintosh; Centre for Geographic Medicine Research Coast, Kilifi, Kenya). Alfredo Mayor (International Centre for Genetic Engineering and Biotechnology, New Delhi, India) described a minimal PfEMP-1 domain involved in CR1 binding and rosetting that could be a target for drug or vaccine intervention. Qijun Chen *et al.* (Karolinska Institut, Stockholm, Sweden) reported that vaccination

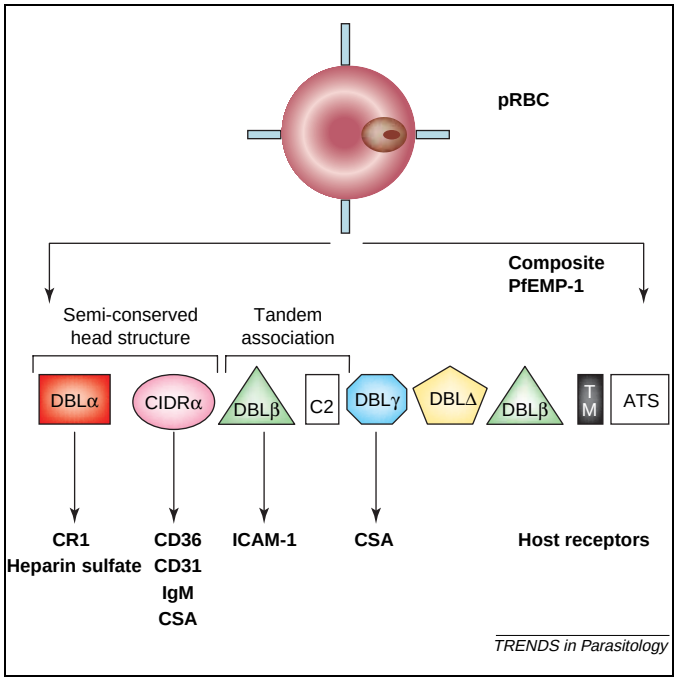

Figure 1. The predicted domain organization and binding properties of the parasitized red blood cell surface protein *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1). The intracellular ATS is highly conserved and anchors the protein to parasite-derived knobs on the RBC surface. The highly variable extracellular region is assembled from three blocks of sequence that share homology: DBL domains, CIDR and C2 inter-domain sequences. The arrows indicate the known binding specificities of each PfEMP-1 region. The binding specificities of domains vary among different PfEMP-1 types. Any single domain from a particular PfEMP-1 might not bind to all of the receptors shown. Abbreviations: ATS, acidic terminal sequence; CIDR, cysteine-rich inter-domain regions; DBL, Duffy-binding-like; PfEMP-1, *Plasmodium falciparum* erythrocyte membrane protein-1; RBC, red blood cell; TM, transmembrane region.

of mice with RNA particles containing a DBLα sequence, encoding multiple adhesive properties, generated antibodies that recognized the pRBC surface, disrupted rosettes and inhibited parasite sequestration in the microvasculature *in vivo*.

Malaria in pregnancy is another area where it might be possible to define a limited set of PfEMP-1 molecules associated with a specific clinical presentation. During pregnancy, pRBCs accumulating within the placenta characteristically adhere to CSA on the surface of placental syncytiotrophoblasts [80,81]. Placental sequestration is also associated with cytoadherence to HA [56]. It has been shown that pRBC binding to CSA is mediated by PfEMP-1 [82] and that parasites binding to CSA express particular variant surface antigens recognized by women in different malaria-endemic areas in a parity-dependant manner, but generally not by men [83]. Michael Duffy (University of Melbourne, Victoria, Australia) reported that the highly conserved *var2csa* gene [84] was the quantitatively dominant *var* gene expressed in different CSA-binding isolates, and was expressed in placental parasites of African women. Crossreactive antibodies could be generated in animals against these isolates (Salenna Elliott; University of Melbourne, Victoria, Australia) and isolates expressing *var2csa* were commonly recognized by antibodies among pregnant women in different geographic regions (James Beeson; University of Melbourne, Victoria, Australia). However, there is also evidence suggesting that there might nonetheless be significant diversity among antigenic determinants expressed by placental pRBCs that might pose challenges for vaccine development.

Prior studies have suggested that not all placental isolates adhere to CSA and HA *in vitro* [54]. Gerhard Winter (Karolinska Institut, Stockholm, Sweden) described a parasite isolate, 3D7S8, that bound to placental tissue but did not adhere to the known placental receptors CSA or HA. The *var* gene expressed by this isolate does not contain a DBLγ domain, previously associated with adhesion to CSA [82]. Antibodies to the isolate were uncommon among immune individuals and were not associated with pregnancy, suggesting that this particular placental-binding variant and expressed *var* is of low immunogenicity or not expressed at high frequencies in populations.

The clinical consequences of infection with *P. vivax* are, in general, less severe than infection with *P. falciparum*, and this is thought to be owing partly to the fact that *P. vivax* does not sequester. However, during pregnancy, *P. vivax* infection has been associated with severe clinical outcomes such as low birth weight, especially in multigravidous women [85]. Kesinee Chotivanich (Mahidol University, Bangkok, Thailand) presented a possible mechanism for this observation: none of 30 *P. vivax* isolates tested bound to CD36, ICAM-1 or TSP *in vitro*, whereas all isolates adhered to CSA and HA, which are receptors implicated in placental *P. falciparum* malaria. However, it should be noted that none of these *P. vivax* isolates were originally from pregnant women. The precise nature of the parasite ligand involved, and any homology it might have to the CSA-binding ligand of *P. falciparum*, PfEMP-1, remains to be seen. At present, sequestration of *P. vivax* has not been observed; however, perhaps the dogma of *P. vivax* being a non-sequestering parasite might need to be revised.

Malarial anaemia

In contrast to the number of studies investigating the pathophysiology of CM, malarial anaemia seems to have been relatively neglected. Of nearly 300 presentations at MAM 2004, very few dealt directly with the pathogenesis of malarial anaemia. The pathogenesis of malarial anaemia is complex and undoubtedly involves multiple processes relating to both the destruction of erythrocytes and their reduced production (reviewed by Ref. [86]).

Increased RBC destruction

In acute malaria, RBCs are directly destroyed by infecting parasites [87]. However, the degree of RBC loss and anaemia cannot be explained solely by this process. Non-parasitized RBCs are also removed from the circulation by complement-mediated lysis and phagocytosis resulting from immune complex deposition and complement activation. Recent studies suggest RBCs from children with severe malarial anaemia have significantly reduced levels of complement regulatory proteins [88], which protect RBCs from destruction by removing circulating immune complexes and regulating the complement activation cascade [89]. These deficiencies resolved following treatment with antimalarials and blood transfusion, which is indicative of the acquired nature of the deficit associated with malaria [88]

Reduced RBC production

During *P. falciparum* infections, reticulocyte levels are inappropriately low [87], reflecting suppression of the normal response of erythropoietin (EPO). Recent work using microarrays to profile gene expression in a murine model of malaria has shown that there was an initial repression of erythroid-associated transcripts both in the spleen, which is a primary erythropoietic site, and in the bone marrow during early infection [90,77,59]. Given that the levels of EPO appear to be suitably raised in cases of severe malarial anaemia [91], a finding that is not universal [92], it is hypothesized that the lesion resulting in such marked dyserythropoiesis lies at or downstream from the EPO receptor [90].

Summary

Over the past 15 years, the field as a whole has begun to move away from polarized views of 'either/or' mechanisms for severe malaria (e.g. physical obstruction versus inflammation) towards the recognition that severe malaria is an extremely complex multi-process and multi-system disorder. Similarly, debates over the relevance of murine models to human malaria show signs of giving way to a more productive interplay between clinical observations and experimental approaches in models and *in vitro*. The new fields of genomics and proteomics offer powerful tools, although also the potential to overwhelm with information. However, in the midst of excitement over all the potential new approaches, it is sobering to remember that no new intervention has had a proven

effect in reducing the mortality of severe malaria in the past 50 years; this is the challenge to be faced.

Acknowledgements

C.L.M. and K.M. are supported by funding from the Wellcome Trust (UK), and J.G.B. is supported by the National Health and Medical Research Council of Australia (Training Award).

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